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EFFECT OF UREA, THIOUREA, PHENYLTHIOUREA AND THIOURACIL ON THE OXYGEN CONSUMPTION OF BLOCKED AND ACTIVE EMBRYONIC CELLS [1]

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Zoological Laboratory, State University of Iowa, Iowa City, Iowa

Introduction

Considerable data on the action of urea, thiourea, thiouracil and related compounds on the intact organism are at hand, especially as regards their action in such phenomena as growth and thyroid function (Lynn, 1948). That thiourea and thiouracil are important in the activity of the thyroid gland has been widely demonstrated both clinically and otherwise. The exact mechanism of their action on cells, however, seems to be less well understood. The fact that certain oxidations, involving cytochrome-cytochrome oxidase or the peroxidase systems, are significant in the reactions of thiourea and thiouracil seems well established (Sadhu, 1948). It becomes of some interest, therefore, to compare the effects of such chemicals upon the respiratory mechanisms of embryonic cells, both in the mitotically active as well as in the resting or blocked condition. The present paper has to do with results of studies on the effects of urea, thiourea, phenylthiourea and thiouracil upon the oxygen uptake of mitotically active and blocked cells of the embryo of the grasshopper, *Melanoplus differentialis*.

Materials and Methods

Eggs of the grasshopper, *Melanoplus differentialis*, were obtained and dissection of embryos carried out as previously noted (Bodine and Boell, 1934). Sterile phosphate-buffered Ringer solution (pH 6.8) was used as the suspending medium for the embryos. All solutions of chemicals (c.p.—urea, thiourea, phenylthiourea, thiouracil [2]) were made up in this buffered Ringer solution. Diapause (blocked) and post-diapause (active) embryos were, from all external appearances, morphologically identical (Bodine and Boell, 1936). Only embryos from eggs

[1] Aided by grant from the National Institute of Health.

[2] Samples of thiouracil were generously donated by the Lederle Laboratory of Pearl River, N. Y.

of known temperature and developmental histories were employed. Diapause eggs were those kept constantly at 25° C. from the time of laying, and which were then confirmed by O_2 uptake determination to be in the blocked state. Post-diapause eggs were those diapause eggs previously kept at 5° C. for periods long enough to remove the block and which were also confirmed by further O_2 consumption tests to be in the active state.

Oxygen determinations were carried out at 25° C. with standard Warburg equipment, using 5 cc. flasks. Control or blank runs on all embryos were for 30 minutes, after which the reagents were added from the side arms and the oxygen consumption rates followed for periods of no less than one hour. Three banks of manometers, 18 in number, were used in individual runs and general averages calculated. Each concentration of reagent was tested many times, so that over-all reading totals for each point represent several hundred determinations. One hundred embryos were used in each flask. In comparing reactions of blocked and active embryos, all determinations on a single lot were carried out simultaneously, so that experimental conditions were kept similar throughout.

Concentrations of reagents were calculated from the quantities added to the vessels and it was found that the degree of effect shown by 50 embryos compared favorably with that for 100 embryos, indicating that no appreciable amounts of the reagents were taken up by the embryos.

Results

Urea

The results of the effects of urea upon the oxygen uptake of both active and blocked cells of embryos are graphically depicted in Figure 1. An inspection of

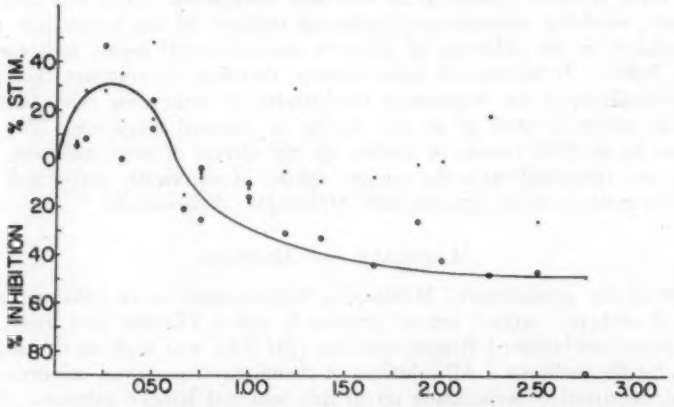

Figure 1. Effect of concentration of urea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration of urea. Solid curve is average for all experiments on blocked and active embryos for low concentrations and for active embryos in high concentrations. Results for a typical experiment are indicated—solid circles for blocked, open circles for active embryos.

this figure shows a marked stimulation of the oxygen consumption with low doses of urea and a corresponding inhibition with higher doses. The general shape of the curve is similar to that found for ethyl carbamate (Bodine and Fitzgerald, 1948), and is perhaps more or less typical of the reaction of this material to many such reagents. No significant differences in the response of blocked and active cells are apparent for the stimulating effects of low concentrations of urea. For higher, inhibitory doses, however, a marked resistance to the reagent is shown by the blocked cells, the mitotically active cells being much more affected by such exposures. Up to concentrations of approximately .2 molar, the inhibitory effects of the urea on the oxygen uptake of blocked cells are small and completely reversible. For the active cells at concentrations of 1.25 molar, one gets about 50 per cent recovery in oxygen consumption rates, while at 2.5 molar the maximum average recovery amounts to but 25 per cent.

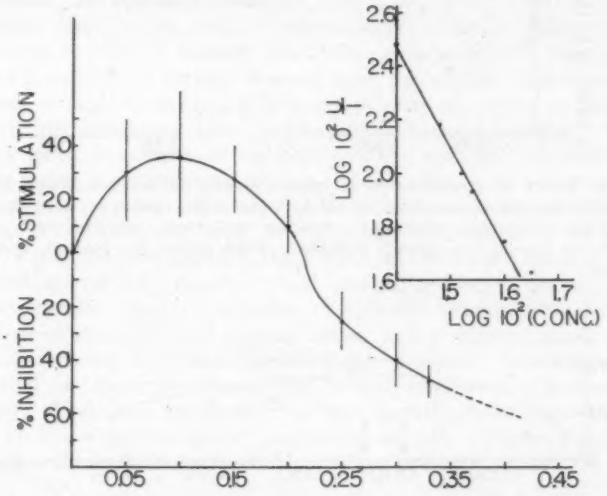

FIGURE 2. Lower left, effect of concentration of thiourea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration of thiourea. Solid curve is average for all experiments. Vertical lines through points represent extent of variation for specific concentrations. Upper right, a log-log plot showing relation between ratio of uninhibited (U) and inhibited (I) respiration and concentration of thiourea. Ordinate, log 10^2 U/I. Abscissa, log 10^2 concentration of thiourea.

A point of some interest as regards the reaction of the embryos, both blocked and active, to urea is that after exposure to the reagent and subsequent washing and suspension in Ringer solution, a decided swelling is observed. The intensity of this swelling seems dependent upon the concentration of urea employed. Such a reaction has been observed only after exposure to urea and no other compound. Further details concerning this reaction will be dealt with in a subsequent report.

Thiourea

The effects of thiourea on the oxygen consumption of cells are qualitatively similar to those found for urea. Results are shown graphically in Figure 2. Marked stimulation of oxygen uptake by low concentrations and inhibition by higher concentrations are invariably produced. No significant differences in response to any concentration of the thiourea by the blocked and active cells are noted. Practically complete recovery in oxygen uptake occurs, except for the highest concentrations of reagent employed. The relative effective concentrations of thiourea, as will be noted from Figure 2, are much lower than for urea.

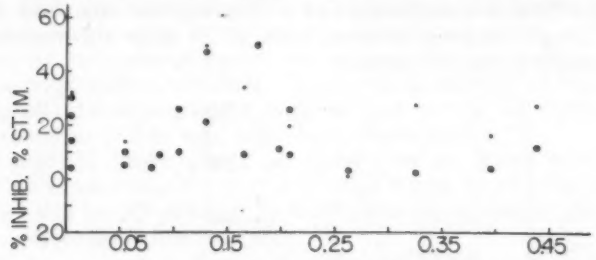

FIGURE 3. Effect of concentration of phenylthiourea on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (top) or inhibition (bottom). Abscissa, molar concentration $\times 10^3$ of phenylthiourea. Results for typical experiments indicated. Solid circles for blocked, open circles for active embryos.

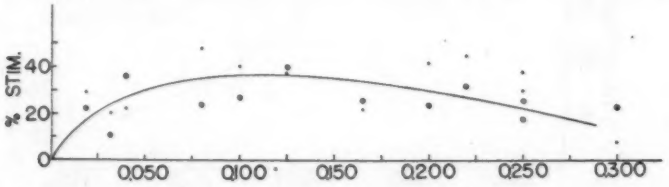

FIGURE 4. Same as Figure 3, but for thiouracil. Solid curve is average for all experiments. Results for typical experiment shown.

Phenylthiourea

The action of phenylthiourea on the oxygen uptake of both blocked and active cells is quite similar to that for urea and thiourea. As indicated in Figure 3, only stimulation of the oxygen uptake has been found for the concentrations employed. Such a result is doubtless due to its low solubility and to the low concentration of the compound used. Complete recovery is found for all concentrations of the reagent.

Thiouracil

Thiouracil, like phenylthiourea, is but slightly soluble and hence only low concentrations of this reagent are available. A marked increase in oxygen uptake

for both blocked and active cells is found (Fig. 4). No significant differences in response for blocked or active cells are noted. When washed and resuspended in Ringer solution, complete recovery in oxygen uptake occurs.

Discussion of Results

The rather marked and similar effects of urea, thiourea, phenylthiourea and thiouracil upon the oxygen uptake of both blocked and active embryonic cells are indeed striking, and several points of general interest in respect to their fundamental action arise. The fact that thiourea and thiouracil have been shown to be involved in the respiratory mechanisms of cells (Sadhu, 1948) would lead one to suspect similar actions on the oxygen uptake mechanisms of the type of cells employed in these investigations.

The oxygen consumption rates of blocked and active embryos (those mitotically blocked or active) are markedly different (Bodine and Boell, 1934). For morphologically similar embryos, the rates of oxygen uptake of active embryos are at least three to four times that of blocked ones. It seems important, therefore, in any discussion of the effects of various reagents upon the oxygen consumption of these forms, to keep in mind these basic differences in rates of oxygen uptake associated with their cellular behavior. Urea is the only compound found to differentially affect the oxygen consumption of the embryos, and then in such high concentrations as to be rather toxic for the active ones. Blocked cells are but little affected by relatively high concentrations of urea, and then in a completely reversible manner. For all other compounds employed, no significant differences in the response of blocked and active cells are found. Any consideration of the effects of these compounds, therefore, will refer equally to both blocked and active cells.

The characteristic curve of response to different concentrations of these reagents—a marked stimulation of oxygen uptake in low concentrations and a similarly marked inhibition in higher concentrations—compares favorably with results found for ethyl carbamate (urethane) and related compounds (Bodine and Fitzgerald, 1948). Urethanes are thought to have marked effects upon the dehydrogenases or carriers of the respiratory mechanisms of cells. Thiourea and thiouracil inhibit certain enzyme systems, probably the oxidase and peroxidase activity of cells (Sadhu, 1948).

Many invertebrates, as is well known, use copper in functions normally taken over in higher forms by iron, for example haemocyanin. Grasshopper embryos, at the stages used in the present work, contain appreciable amounts of copper (approximately 0.025 μgm. per embryo [3]), and it is reasonable to assume that it functions for them in enzyme systems in a manner comparable to other cations for higher forms. It is also well known that copper has marked action upon many enzyme systems, and especially sulfhydryl-containing enzymes (Barron and Singer, 1945). As a specific example, it has been rather clearly shown that copper markedly inhibits the hemolysis of red blood cells in isosmotic glycerol solutions (Jacobs and Carson, 1934). Explanations of such phenomena suggest the action of copper on a sulfhydryl-containing enzyme system located in the red blood cell (LeFevre, 1948). It becomes of some interest, therefore, to examine the possibility of the part played by the contained copper of grasshopper embryos in their reactions to the reagents employed.

[3] Unpublished data from this laboratory.

It can readily be shown that tyrosinase, a copper-containing enzyme, isolated from the grasshopper egg, is markedly inhibited by thiourea.[4] Such a reaction for this enzyme is well known, and the explanations based upon a copper-thiourea reaction seem well founded (Denny, 1943). The possibility of such copper combinations with the other reagents employed seems worthy of comment.

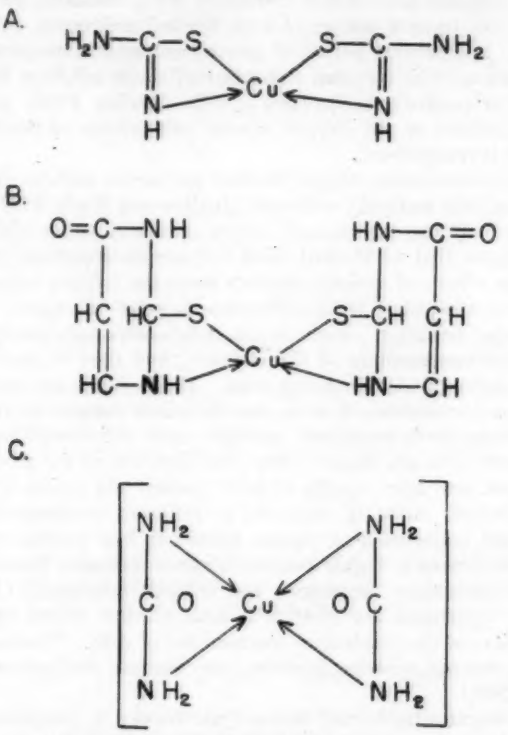

FIGURE 5. Scheme for possible combinations of reagents with copper. A = thiourea, B = thiouracil, C = urea.

For thiourea and thiouracil (A and B of Fig. 5[5]), there is some evidence for the existence of the copper combination as indicated. Theoretically, there is no reason for which a complex copper-urea ion could not exist for urea (C of Fig. 5) in a form essentially as shown. Recently it has been pointed out by LeFevre (1948) that the molecular concentration of reactivating substances (containing SH groups) had to equal, or exceed by one to two times, the concentration of the inhibitor (copper) used in the case of hemolysis of red blood cells. Experiments carried out in

[4] Unpublished data from this laboratory.

[5] Acknowledgment is made to Professors George Glockler and Ralph L. Schriner of the Department of Chemistry for their suggestions in this problem.

connection with the present investigations, using concentration of copper and of thiourea in such proportions, corroborate the findings of LeFevre.

The effects of thiourea and thiouracil on cell respiration and cell division have been variously reported; in some cases inhibition, while in others stimulating responses, are described (Fearon, 1942). Embryos of the grasshopper while in the blocked condition have been shown to lack mitotic activity and to have rates of oxygen uptake at a low constant level. When active, mitosis is always present and rates of oxygen uptake are increasingly higher than for the blocked or inactive states. Such naturally-occurring cellular conditions, therefore, make possible checks on the parts they play in reactions to various reagents. Urea is the only compound with which a marked and significant difference in response between blocked and active cells is noted. Blocked cells are extremely resistant to this compound while active ones are markedly affected, and usually in an irreversible manner. No explanation for such a basic difference in response is at hand.

Published data concerning the effects of urea, etc., on the respiration of cells are fragmentary or almost completely lacking, so that comparisons between copper-containing cells like those used in the present experiments, and those for higher forms, seem practically impossible. The bacteriostatic action of urea alone and in combinations with the sulfonamides seems well established (Kirby, 1943). Alkyl ureas have been shown to depress certain respiratory enzymes (dehydrogenases, coenzymes) (Grant and Krantz, 1942). The effects of thiourea as well as of thiouracil upon such basic phenomena as mitosis, however, seem at present rather confused (Paschkis, Cantarow, Rakoff and Rothenberg, 1945). The effects of thiourea and thiouracil upon the growth of amphibians, as well as upon the thyroid gland itself, are rather clear and well defined (Lynn, 1948).

The stimulating effects of low concentrations of all reagents, although typical, are for the present not readily explained. Permeability and other factors have been suggested for similar phenomena in other forms (Lillie, 1916). Lack of inhibition of oxygen uptake in the case of phenylthiourea and thiouracil appears to be the result of the low solubility of these compounds, and thus for them solubility becomes the limiting factor.

The most regular and consistent responses for any reagent used are those for thiourea, and even here solubility of the compound becomes the limiting factor in a complete analysis of its effect on oxygen consumption. Responses to it for blocked and active cells are not significantly different. An analysis of its inhibitory effects according to the law of mass action, as pointed out by Fisher and others (Fisher and Henry, 1943), would suggest that its action may be similar to that for urethane and that a single respiratory mechanism may be involved (Fig. 2).

Summary and Conclusions

1. The action of urea, thiourea, phenylthiourea and thiouracil on the oxygen uptake of the blocked and active cells of the embryo of the grasshopper, *Melanoplus differentialis*, has been studied.

2. In general, low concentrations of these reagents produce stimulation, while higher concentrations produce inhibition of the oxygen consumption of both blocked and active cells.

3. Urea alone produces a differential effect, in that blocked embryos are little affected by high concentrations while active embryos are irreversibly inhibited.

4. The relative effectiveness of the compounds upon the oxygen consumption of blocked and active cells is: thiouracil > phenylthiourea > thiourea > urea; recovery seems to work in the opposite order.

5. Suggestions are made as to the possible significance of copper in determining the basic reaction of these compounds.

ACKNOWLEDGMENT

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THE EFFECTS OF ELECTROLYTES AND SUGARS ON THE ERYTHROCYTES OF THE TURTLE, CHELYDRA SERPENTINA[1]

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The erythrocytes of the snapping turtle, *Chelydra serpentina,* exhibit a particular sensitivity to the lack of calcium and will hemolyze in electrolyte solutions which do not contain this ion, though not in pure glucose (Lyman, 1945). The absence of hemolysis reported for glucose is presumed to be due to impermeability to the glucose molecule because of its size. If this is true, sugars of smaller molecular volume might prove hemolytic. Two factors other than size may also have an influence. From Gibbs-Donnan equilibrium relationships, changes in membrane structure may be expected when a non-electrolyte replaces an electrolyte medium, all sugars having an equivalent action in this respect. In addition, sugars might affect the cell membrane by direct chemical action, and such effects may be characteristic for the individual sugars. With regard to the hemolytic action of calcium-free electrolyte solutions on Chelydra erythrocytes, it is not known to what extent electrolytes differ in their action, nor in what manner an alteration of the hydrogen ion concentration of any given solution may influence the hemolytic process. These various factors have been considered in the present study.

The results show that hemolysis occurring in calcium-free electrolyte solutions is influenced by the ionic composition of the medium. Moreover, by suitable adjustment of the hydrogen ion concentration, the integrity of the cell membrane may be maintained for short periods even in the absence of calcium. In an examination of the effects of isosmotic solutions of various sugars, striking differences were found, some being hemolytic and others not. Agglutination and hemolytic reactions observed in certain sugars indicate that these compounds are not inert, but produce a definite alteration of the cell membrane.

Material and Methods

Blood (0.5 – 1.0 ml.) of *C. serpentina* drawn without anticoagulant was washed twice in 40 ml. of frog Ringer and suspended in Ringer. In determining the rate of hemolysis, washed cells were centrifuged briefly; the supernate was very carefully removed, and 5.0 ml. of experimental solution added to give a cell concentration of approximately 1 : 100. The optical density was measured within the first minute and at intervals with a Fisher electrophotometer (Wilbur and Collier, 1943). A Beckman spectrophotometer, made available through the kindness of Dr. W. J. Dann, was used for determinations of the optical density of hemoglobin solutions and for the hemoglobin spectrum of turtle blood.

[1] Aided by a grant from the Duke University Research Council.

Hematocrit tests showed frog Ringer to be approximately isotonic with slight variations between individuals. Experimental solutions were made isosmotic with 0.125 molal NaCl. Double distilled water was used throughout. Galactose, xylose and arabinose were Pfanstiehl brand and were free of calcium. NaCl was Merck Reagent For Biological Work. Other chemicals were reagent grade and were not further purified.

We wish to thank Dr. M. H. Jacobs and Dr. H. B. Collier for their helpful suggestions and Mr. N. G. Anderson and Mr. R. L. Rigsbee for photographic work.

Results

Electrolytes

The observation of Lyman (1945) that erythrocytes of *C. serpentina* will hemolyze in isotonic Ca-free salt solutions was readily confirmed. Moreover the course of hemolysis in isosmotic NaCl, for example, could be arrested by the addition of a small amount of isosmotic $CaCl_2$ solution to the hemolyzing suspension. However, in preliminary experiments it became apparent that the rate of hemolysis varies with the cation and anion employed and also with the hydrogen ion concentration.

The effect of different cations was examined by following the course of hemolysis in buffered and unbuffered isosmotic solutions of NaCl, KCl, $MgCl_2$ and $CaCl_2$. Hemolysis was always most rapid in KCl, followed by NaCl, slower in $MgCl_2$ (Figs. 1 and 2), and completely absent in $CaCl_2$.

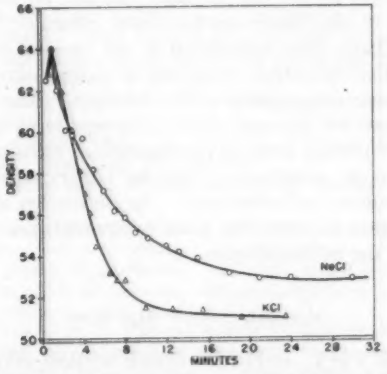

FIGURE 1. Hemolysis of Chelydra erythrocytes in 0.125 molal NaCl and 0.126 molal KCl. 29° C. pH 7.41. Solutions buffered with corresponding isosmotic phosphate in the proportion 9:1. The initial rise in the curves indicates preliminary shrinkage probably due to slight hypertonicity. The final values for optical density represent complete hemolysis for both solutions, the difference apparently resulting from difference in opacity of the ghosts.

The rate of hemolysis in any given electrolyte solution was always greater in an alkaline than in an acid solution. This could be shown by adjusting the pH with HCl or the hydroxide of the cation being studied, or by use of phosphate buffers. Figure 3 illustrates the effect in the case of KCl buffered with isosmotic phosphate.

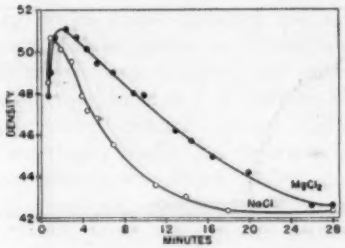

FIGURE 2. Hemolysis of Chelydra erythrocytes in 0.125 molal NaCl and 0.088 molal $MgCl_2$. 28° C. pH 7.41. Solutions buffered with 0.1 molal sodium phosphate 9:1.

NaCl behaved similarly. The rate showed little change within the range pH 7.8 to pH 6.7 but was definitely decreased at pH 5.9 to pH 6.1. The inhibitory effect of acidity was demonstrated in another manner (Fig. 4). Cells were placed in NaCl buffered to pH 7.0 with a trace of phosphate. After hemolysis was well under way acidified NaCl was added. Hemolysis was quickly arrested. On the addition of alkaline NaCl hemolysis was resumed at the original rate, indicating reversibility of the inhibition. If cells remain in the acid NaCl longer than about 10 minutes, hemolysis will be resumed at a very slow rate. When the pH is restored to the alkaline range after 50 minutes the former rapid rate is regained.

The effects of the cyanide and citrate of sodium and potassium were compared with the corresponding chloride at pH 7.25 or 7.4. Isosmotic mixtures of chloride and cyanide containing 0.019 molal cyanide exhibited a hemolytic action similar to that of isosmotic chloride. The same results were obtained with cells exposed to

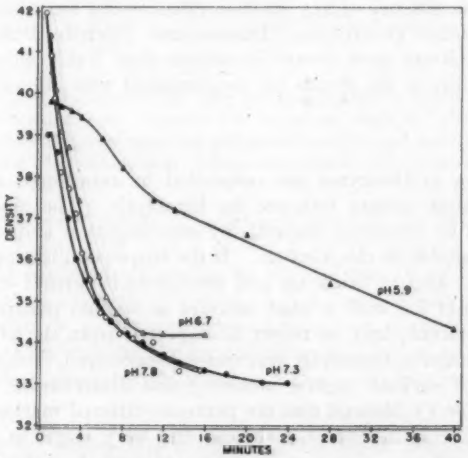

FIGURE 3. Hemolysis of Chelydra erythrocytes in 0.126 molal KCl buffered with 0.1 molal potassium phosphate 9:1. 28° C.

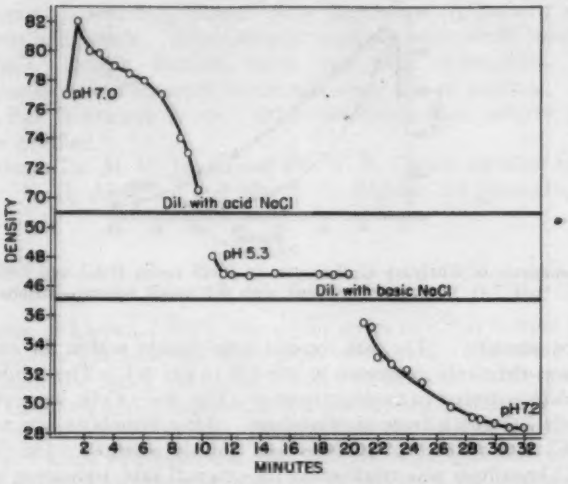

FIGURE 4. Effect of acidity on hemolysis of Chelydra erythrocytes in 0.125 molal NaCl. Ten minutes after the erythrocytes were mixed with 4.0 ml. NaCl solution (pH 7.0), and during the course of rapid hemolysis, 3.0 ml. of acid NaCl was added bringing the pH to 5.3. Hemolysis was arrested within one minute. Addition of 1.9 ml. of alkaline NaCl at 20 minutes raised the pH and hemolysis continued immediately. Dilution gave a marked change in optical density as indicated. The initial rise in the curve indicates shrinkage probably due to hypertonicity. 21° C.

the same concentration of cyanide in Ringer for 30 minutes before adding the hemolytic solution. The absence of any marked effect is not surprising in view of previous work with other electrolytes (Davson and Danielli, 1938; Hunter, 1947). Isosmotic sodium citrate gave slower hemolysis than NaCl, which may be the result of impermeability to the citrate ion as compared with chloride (Jacobs, 1940).

Sugars

When Chelydra erythrocytes are suspended in unbuffered isosmotic dextrose solution, agglutination occurs followed by hemolysis. The amount of hemolysis can be determined by removing the cells by centrifugation and measuring the concentration of hemoglobin in the solution. If the suspension is pipetted up and down, the masses of cells tend to break up and hemolysis is further increased. In dextrose buffered at pH 7.5 with a small amount of sodium phosphate, agglutination was no longer apparent; and, as might be expected from the effect of pH on hemolysis in salt solutions, hemolysis was more pronounced.[2] A comparison of the hemolytic action of various sugars, including one disaccharide, two hexoses, and two pentoses (Table I), showed that the pentoses differed markedly from the other sugars, hemolysis being absent in arabinose and very slight in xylose. Dextrose gave complete hemolysis. On centrifugation of such hemolyzing suspensions, a

[2] The buffer may well have an effect as an electrolyte.

jelly-like mass containing nuclei, many distorted and a few normal erythrocytes, was found. This contrasts with hemolysis in electrolytes in which normal ghosts were present, with about 50 per cent of the ghosts sphering in isosmotic $MgCl_2$.

Observations on agglutination indicate that differences exist between sugars in this respect as well as in their hemolytic action. Cells (0.07 – 0.08 ml.) suspended in Ringer were centrifuged briefly; the supernate was carefully removed and 8.5 ml. of isosmotic sugar solution was added. After stirring to give uniform cell distribution, the suspension was left undisturbed.[3] Results were similar for three individuals (Table I). While strict comparisons between certain sugars cannot be made because of differences in pH, it is apparent that all sugars cause agglutination, but all are not equivalent. Moreover, the effects of agglutination do not parallel hemolytic actions. So, for example, sucrose is hemolytic and weakly agglutinating; arabinose, on the other hand, has a greater agglutinating action without producing hemolysis.

TABLE I

Hemolysis and agglutination in isosmotic sugar solutions

Cells were removed from solutions after 3 hours and the optical density of the supernate was measured at 576 millimicrons.* pH 7.5-25° C. Blood from three turtles.

	Hemolysis		Agglutination	
	Sample 1	Sample 2		pH
dextrose	0.630	0.510	++++	5.4
sucrose	0.344	0.378	+	5.7
d-galactose	0.325	0.163	+	4.5
d-xylose	0.087	0.017	++++	4.4
l-arabinose	0.007	0.005	+++	5.7
Ringer	0.017	0.014	0	5.7

* After initial mixing the suspensions were left undisturbed to minimize hemolysis and then centrifuged. Centrifugation may increase the hemolysis slightly. (Collier, 1948. Personal communication.) The absorption spectrum was determined for one sample of blood, and maxima were located at 543 and 576 millimicrons. These agree closely with values given for human blood.

The influence of electrolytes on agglutination may be shown by adding one part of isosmotic NaCl, KCl, $MgCl_2$ or $CaCl_2$ to 14 parts of isosmotic dextrose. Agglutination was inhibited somewhat by the addition of electrolytes in all cases and was less pronounced in the presence of NaCl and $MgCl_2$ than with the other salts (Fig. 5). But in no instance was agglutination completely prevented. Microscopic observations indicate similar differences (Figs. 6 and 7). Quantitative aspects of this effect, previously studied on other cells (Radsma, 1918), have not been investigated.

[3] Sedimentation rate fails to give a measure of the extent of agglutination in this blood inasmuch as some cells do not agglutinate and therefore sediment relatively slowly, whereas the agglutinated masses in the same suspension fall rapidly or adhere to the wall of the tube. Adherence to the wall is observed in vertical tubes of 1.0 cm. or more in diameter.

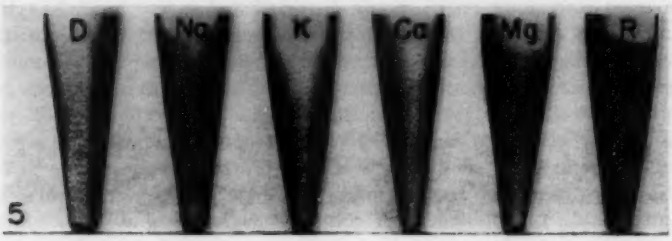

FIGURE 5.

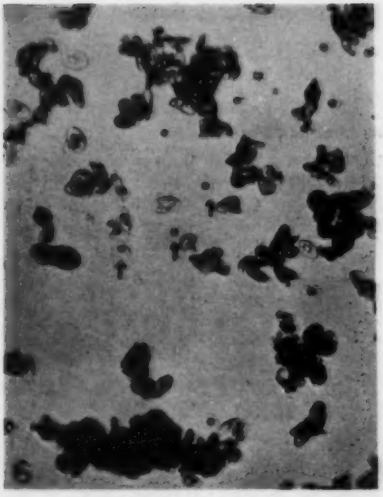

FIGURE 6.

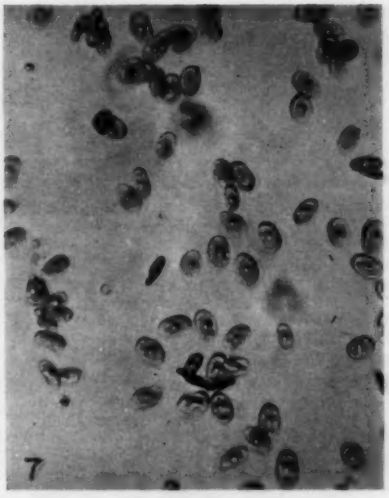

FIGURE 7.

FIGURE 5. Effect of salts on agglutination of washed Chelydra erythrocytes in dextrose solution. Isosmotic salt solutions were added to unbuffered dextrose (0.23 molal) in the proportion 1:14. D—dextrose; Na—NaCl + dextrose; K—KCl + dextrose; Ca—$CaCl_2$ + dextrose; Mg—$MgCl_2$ + dextrose; R—Ringer (unbuffered). After initial mixing tubes were left undisturbed. 26.5° C.

FIGURE 6. Chelydra erythrocytes in 0.23 molal unbuffered dextrose. Washed cells were mixed with dextrose solution and placed immediately on a slide without coverslip. Arrows indicate ghosts.

FIGURE 7. Chelydra erythrocytes in 0.23 molal unbuffered dextrose with isosmotic NaCl 14:1. Shrinkage may be noted.

DISCUSSION

Consideration of the mode of action of electrolytes and non-electrolytes on Chelydra erythrocytes is complicated by the fact that related substances may be strikingly different in their actions. Thus, no single scheme will serve to explain completely the action of sugars, nor can cation effects be interpreted adequately in

terms of valence. The latter is in contrast to human erythrocytes, in which ions of the same valence are alike in preventing loss of salts from cells in sucrose solution (Wilbrandt, 1940).

The effect of calcium in maintaining the normal permeability characteristics of the cell may be considered in relation to (1) the thickness of the ionic double layer and the adhesion of membrane components, and (2) crossbinding of anions within the membrane (Danielli, 1937, 1943). In both respects calcium has a more pronounced action than sodium or potassium. Following the Gibbs-Donnan equilibrium, with a change of medium from Ringer to isosmotic NaCl or KCl as was done in the present experiments, there will be a replacement of calcium in the surface layer. Compactness of the membrane will be decreased, which may in turn lead to an increase in permeability resulting in swelling and hemolysis. Such an increase in volume preceding hemolysis in these solutions may be readily observed under the microscope. Additional assumptions will be necessary to explain such differences as those found between the effects of calcium and magnesium on hemolysis and on sphering of ghost cells.

With sugar solution as the medium, the salt concentration essentially zero, and a constant anion concentration in the cell surface, there is to be expected from the Gibbs-Donnan equilibrium a decrease in membrane concentration of mono- and divalent metal ions, and an increase in surface acidity, which has been thought to be of sufficient magnitude to alter the proteins of the membrane and accordingly cell permeability (Danielli, 1937; Wilbrandt, 1940). However, in this cell an increase in acidity of the medium stabilizes the membrane, as shown by the acid inhibition of hemolysis in both electrolytes and non-electrolytes. At the same time the loss of metal ions would result in increased repulsive forces within the membrane, giving greater distances between molecules and an increase in permeability (Danielli, 1943). Even though it is assumed that the net effect favors an increased permeability, the present results are not completely explained inasmuch as all sugars should behave similarly, whereas some have been shown to be hemolytic while others are not.

Other factors to be considered are a differential permeability to sugars and effects of individual sugars on membrane structure. Any differences in permeability which may exist must involve factors other than molecular volume since this does not correlate with hemolytic action. So, for example, sugars which were least hemolytic (pentoses) have the smallest molecular volume. (See also Ulrich, 1934.) The agglutination of cells indicates an alteration of the cell surface by the sugar, the degree to which this occurs depending upon the particular sugar and ionic composition of the medium. Further, hemolysis in sugar, contrary to the results in electrolyte solutions, was characterized by disintegration of many of the cells, again pointing to a direct action on membrane structure.

The effect of acidity in decreasing hemolysis obtained with electrolyte solutions suggests that molecular rearrangements within the membrane may in part compensate for the lack of calcium. The fact that the erythrocyte is stable in certain Ca-free non-electrolytes indicates that it is not the absence of calcium *per se* which causes hemolysis in electrolytes, but rather the effect of other cations which may replace the calcium of the cell membrane and so increase its permeability. The cell, then, would be sensitive to lack of calcium only because of the ready replacement of its calcium by other cations.

SUMMARY

1. The comparative hemolytic rates of *Chelydra serpentina* erythrocytes in isosmotic salt solutions as measured photometrically were KCl > NaCl > $MgCl_2$, and NaCl > Na_3 citrate. Hemolysis in cyanide (0.019 molal) was similar to that in chloride. No hemolysis occurred in isosmotic $CaCl_2$ and the addition of $CaCl_2$ to cells hemolyzing in Ca-free electrolyte solutions arrested hemolysis at once.

2. Hemolysis in sodium and potassium solutions was greatly retarded at about pH 6 and below.

3. The hemolytic potency of isosmotic sugar solutions (pH 7.4) was found to be: dextrose > sucrose > d-galactose > d-xylose with complete hemolysis in dextrose and none in l-arabinose in three hours.

4. Sugar hemolysis was accompanied by abnormal shape changes and disintegration of cells, whereas in Ca-free electrolyte solutions "normal" ghosts were found.

5. Agglutination occurred in unbuffered isosmotic sugar solutions, the extent depending upon the particular sugar. Agglutinating action was not correlated with hemolytic potency.

6. Results of experiments on the hemolytic and agglutinating properties of sugars indicate that certain sugars are not inert but have a definite action on the cell surface.

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EXPERIMENTS ON THE DETERMINATION AND DIFFERENTIATION OF SEX IN THE BOPYRID STEGOPHRYXUS HYPTIUS THOMPSON [1]

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One of the great controversies related to the Bopyridae (a family of isopod crustaceans, suborder Epicaridea), and one that has broader biological implications as well, is the question of sex-determination. Is sex already determined in the larval stage, or does the fate of the larva (i.e. whether it turns into a female or a male) depend on environmental influences? Can such external factors as the position the larva occupies or the nourishment it receives bring about sex reversal in an already sex-determined larva?

The chief views expressed in the past regarding this question are as follows:

1. Giard and Bonnier (1887) maintained that in the Bopyridae all free-swimming larvae are males. The first larva, however, that invades a particular host undergoes sex reversal and transforms into a large female without ever having functioned as a male. The next to come settles on this female and metamorphoses into an adult dwarf male which fertilizes the former.

2. Smith (1909) and Goldschmidt (1920) stated that all the species of Epicaridea, Bopyrina as well as Cryptoniscina, are protandric hermaphrodites, each individual being male while in a larval state, and then losing its male organization and becoming female as the parasitic habit is assumed. The females, therefore, result from males that have already functioned as males.

3. Hiraiwa (1936) believed that the free-swimming larvae are not males but are sexually undifferentiated, although the sex is already predetermined. Differentiation follows fixation, but is probably not due to environmental factors.

4. Recently, Caullery (1941), impressed by the influence of association on sexuality as exhibited in such animals as Bonellia, Crepidula, and Ophyotrocha, made the suggestion that the sexes may not be fixed from the start, but that direct parasitism of a larva on a host entails differentiation into a female, and indirect parasitism, through the intermediary of a female on which it is stationed, entails differentiation into a male. Lacking direct evidence, however, he suggested an experimental approach to test the validity of this theory. He advised collecting the newly-arrived cryptoniscid larvae that can frequently be found in the brood pouch of a female bopyrid—larvae which according to this view would evolve into typical males under the influence of the environment—and placing them in contact with young crabs not yet parasitized. Caullery thought it probable that these larvae would fix to the crab and become females.

These suggestions of Caullery moved the writer to undertake a series of experiments with the larvae of *Stegophryxus hyptius* Thompson, an ectoparasite of the

[1] Supported in part by a grant from The Catholic University of America Research Fund.

hermit crab *Pagurus longicarpus* Say. This work was carried on in the summer of 1946 at the Marine Biological Laboratory, Woods Hole, Mass. In addition to the experiment suggested by Caullery, reciprocal experiments were undertaken in which presumptive female larvae were taken from the host crab and transferred to the brood pouch of a female bopyrid to test the suspected masculinizing influence of the female on cryptonisci that attach to her body.

The present paper reporting on this work was ready for publication when the writer's attention was drawn to an article in Italian by Reverberi and Pitotti, which, although it appeared in 1942, had not been mentioned in the abstracting journals until 1947. This paper provides the first experimental verification of any of the proposed sex-determination theories with reference to the Bopyridae. The authors, working with *Ione thoracica* Montagu, showed that the control of sex-determination is environmental rather than genetic. However, since there are several points of difference between the biological cycles and sex phenomena of Ione and Stegophryxus, it was decided not to alter the present paper as originally written, but in the discussion and footnotes to draw a comparison between the results reported by Reverberi and Pitotti and our own.

Life Cycle of Stegophryxus

Only about 1.5 per cent of *Pagurus longicarpus* at Woods Hole are parasitized by *Stegophryxus hyptius*. Thompson (1901), in his original description of the species, gave an account of the morphology of the adult female, adult male and some of the immature forms, but the life cycle has heretofore not been discussed.

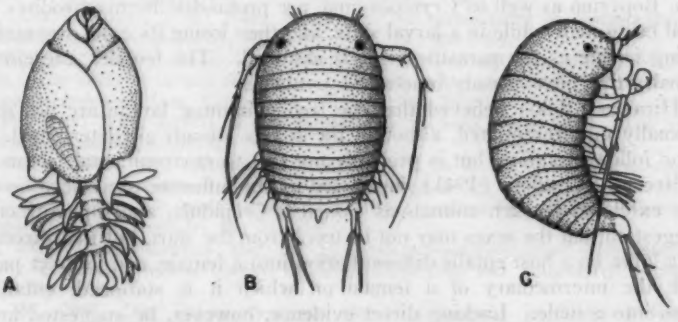

Figure 1. Adult female and epicaridium larva of *Stegophryxus hyptius*.
A. Ventral view of adult female. The dwarf male, although not visible externally, is shown within the brood pouch by a dotted outline to indicate its position and relative size. × 5. B. The epicaridium or first larval stage shown in dorsal view. × 120. C. Lateral view of epicaridium larva. × 120.

The female Stegophryxus, as is the case in all bopyrids, is much larger than the male (Fig. 1A). It occurs on the abdomen of the hermit crab, to which it is attached, back downward, by its mandibles and legs. Its thorax is concealed ventrally by an enormous brood pouch, made up of five pairs of thin brood plates.

Within this brood pouch lies the slender dwarf male, whose function is not that of inseminating the female and then quitting her, but of remaining in readiness to fertilize the successive batches of eggs that are released into the brood pouch during the female's productive life. These eggs, within two weeks after fertilization, develop into first stage larvae that leave the mother and swim off. After an interval of about five days, the marsupium is again filled with eggs and another brood begins embryonic development.

The first larval stage is known as the *epicaridium*. It is a short, broad, semi-barrel-shaped larva (Fig. 1B) with sub-chelate pereiopods for clinging and with pleopods in the form of swimming organs. The epicaridium of Stegophryxus measures about 270 μ in length, 150 μ in breadth, and 120 μ in depth (not including the appendages). In this stage the young of Stegophryxus escape from the brood pouch and swim off as plankton organisms. In the laboratory, they quickly rise to the surface of the water and remain there floating or swimming about for days.

The subsequent history of the epicarid larva has not been investigated in Stegophryxus. It may attach to a pelagic copepod, undergo a molt, and become a *microniscus* larva, which, after feeding on the copepod, will eventually transform into a new larval stage known as the *cryptoniscus* that swims off to seek the definitive host. This type of development is known to occur in some of the Epicaridea (Sars, 1899; Caullery, 1907; Caroli, 1928; Reverberi and Pitotti, 1942). Or the epicarid larva may develop directly into a cryptoniscus larva, an abbreviated type of development which Hiraiwa (1936) believes is the case in most Bopyridae. We postulate the first alternative in the case of Stegophryxus because of the great difference in size between its epicaridium and cryptoniscus stages, a difference which can only be accounted for by assuming the existence of an intervening stage.

At any rate, however arrived at, the earliest larval stage of Stegophryxus that we find on the crab is the cryptoniscus. In this stage (Fig. 2) the parasite is typically isopod in its characteristics. It has an elongated body, dorsoventrally compressed, segmented and well chitinized. There are seven pairs of thoracic appendages (as compared with six pairs in the epicaridium) all similar in form, six pairs of uniramous natatory pleopods, and one pair of biramous uropods. The cryptoniscus measures about 680 μ in length, being therefore about two and one-half times longer than the epicaridium.

We have been able to distinguish three phases in the life of the cryptoniscus larva on the basis of color pattern which we shall designate as (1) the brown chromatophore phase, (2) the black chromatophore phase, and (3) the striped contracted phase.

The youngest cryptonisci, those that have recently settled on a crab, have a profusion of dark brown expanded chromatophores that cover the dorsal surface of the body in such a way as to leave an uncolored portion that resembles a cross (Fig. 2A). These chromatophores are present laterally on the head and segments 1, 4, 5, 6, 7, and 8; are present centrally as well as laterally on segments 12 and 13 and on the uropods; and are entirely absent from segments 2, 3, 9, 10, and 11. The general body color is pale yellowish and is due to another system of chromatophores, which are scattered over the integument without definite plan. The eyes are reddish brown.

In phase 2, the light colored cross-shaped pattern remains much as before, but most of the areas formerly occupied by brown chromatophores are now occupied

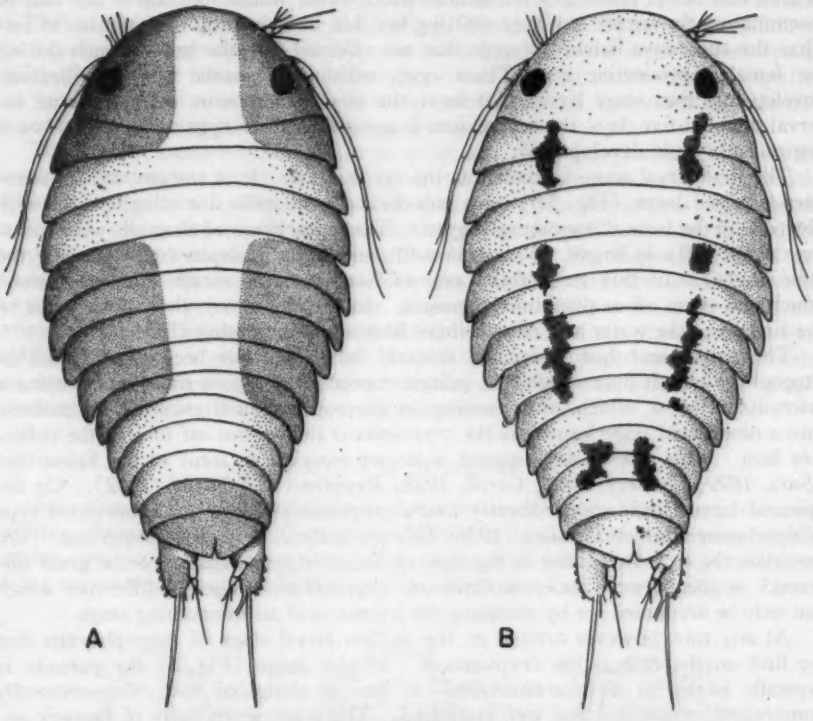

FIGURE 2. The cryptoniscus larva of *Stegophryxus hyptius*.
A. Young larva showing color pattern when in phase 1. The light cross-shaped area is devoid of chromatophores. B. Older cryptoniscus in phase 3 with contracted melanophores forming an interrupted stripe on each side.

by expanded black chromatophores. Their distribution is as follows: present laterally on the head and segments 1, 4, 5, 6, 7, and 8; present centrally on segments 11 and 12; absent from segments 2, 3, 9, 10, 13 and the uropods. The eyes have also become black. The yellow chromatophores are now more noticeable and have become restricted to segments 1 to 11 inclusive where they are present laterally.

In phase 3 (Fig. 2B) the black chromatophores are much fewer in number and are all in the contracted state. They form a broken chain on each side of the body about midway between the center and margin of the dorsal surface, reaching from segment 1 to segment 8 inclusive, but absent on segment 3. On segments 10 and 11 there are a few black chromatophores centrally located. Yellow chromatophores are intermingled with the black in the same chain but extend from segment 1 to segment 11. The eyes are black. In this stage the cryptoniscus is ready for the molt which will transform it into a juvenile female of the first postlarval stage.

No structural differences have been detected in these three cryptoniscid stages. Since neither the brown nor the black chromatophores lose their color in alcohol, they are no doubt melanophores which presumably differ only in the amount of melanin present.

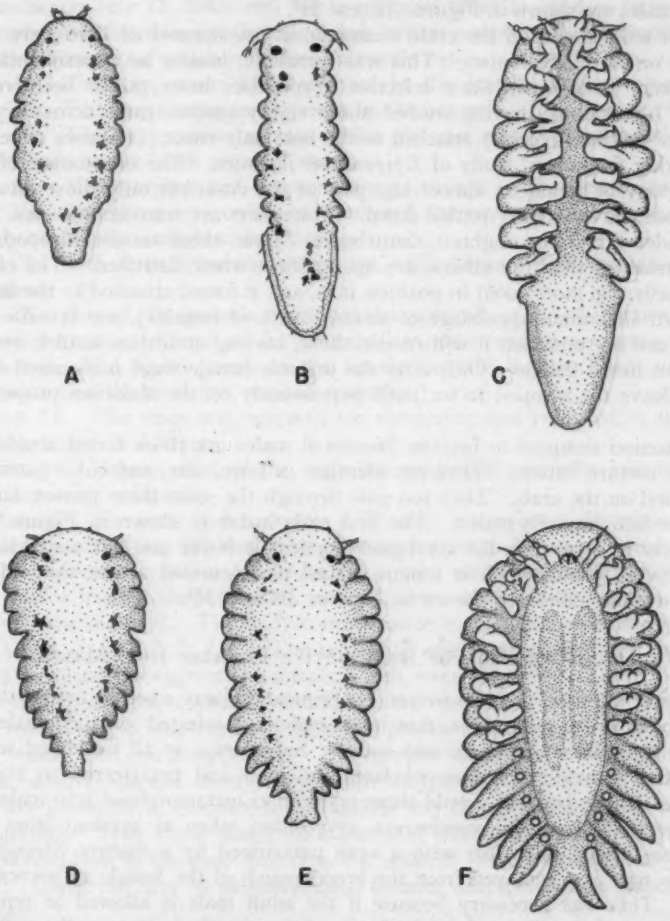

FIGURE 3. Juvenile males of *Stegophryxus hyptius* compared with juvenile females of the same species and same degree of development.

A. Juvenile male in first post-cryptoniscid instar. Specimen drawn measured 0.85 mm. Dorsal view. B. Older juvenile male measuring 1.07 mm. in length. Dorsal view. C. More advanced juvenile male measuring 1.4 mm. in length. Ventral view. D. Juvenile female in first post-cryptoniscid instar. Specimen drawn measured 0.85 mm. Dorsal view. E. Older juvenile female, 1.01 mm. in length. Dorsal view. F. More advanced juvenile female measuring 1.3 mm. in length. Ventral view.

The juvenile female into which the cryptoniscus transforms is shown in Figure 3D. It is broader than the cryptoniscus and the pleon lacks uropods and possesses only stump-like rudiments of pleopods. The terminal segment is extended into a tail-like outgrowth ending in a shallow notch. The animal is whitish with color pattern differing little from that of the last stage cryptoniscus. More advanced juvenile females are shown in Figures 3E and 3F.

About ten per cent of the crabs examined in the summer of 1946 were infested with one or more cryptonisci. This was fortunate, insofar as experimental possibilities were concerned, since infective cryptonisci have rarely been reported. Bonnier, for example, having studied about eighty species, came across cryptoniscus larvae that had recently attached to the host only twice. Hiraiwa never found them during five years' study of *Epipenaeon japonica*. The cryptonisci of Stegophryxus may be found on almost any part of the crab, but only those attached to the pleopods have actually settled down. The others are transients or new arrivals that wander off at the slightest disturbance. Even those on the pleopods, while more permanent than the others, are apt to leave when disturbed. The older the cryptoniscus, the more fixed in position it is, and if found attached to the last pleopod (third abdominal appendage of males, fourth of females), the favorite resting site, one can presume that it will remain there, barring accidents, until it eventually transforms into a female. Only after the juvenile female stage is assumed does the parasite leave the pleopod to fix itself permanently on the abdomen proper of the host.

Cryptonisci destined to become functional males are those found attached to a young or mature female. They are identical in form, size, and color pattern with those found on the crab. They too pass through the same three phases, but metamorphose into juvenile males. The first male instar is shown in Figure 3A. It is narrower in form than the corresponding female instar and has a strikingly different type of pleon which is tongue-shaped and rounded at the tip. More advanced juvenile males are shown in Figures 3B and 3C.

Transformation of Presumptive Females Into Males

The first question to be answered experimentally was whether or not the cryptonisci found on normal crabs, that is on crabs not infested with a female Stegophryxus, could be transformed into males. Such larvae in all likelihood would be presumptive females. If removed from the crab and transferred to the brood pouch of a female bopyrid, would these cryptonisci metamorphose into males?

I. In the first series of experiments, cryptonisci taken at random from normal crabs were placed in a dish with a crab parasitized by a mature Stegophryxus. The male was first removed from the brood pouch of the female to prevent interference. This was necessary because if the adult male is allowed to remain, although cryptonisci will attach as freely as when no male is present, they will enjoy only a relatively brief period of attachment before they are driven off.

Four experiments of this type were conducted. In all cases the greater proportion of the cryptonisci attached to the female bopyrid and the greater proportion likewise entered the juvenile male phase. But, after varying lengths of residence in the brood pouch and correspondingly varied degrees of attainment of the male phase, all but one out of each lot eventually deserted the female. The one that re-

mained in unchallenged possession eventually became a mature male, and in cases where the experiment was continued long enough this male functioned as such and successfully fertilized the eggs of its consort which then developed normally into epicaridium larvae.

It will be sufficient to cite one experiment of this series in detail. This experiment was begun July 13, 1946 with five cryptonisci taken at random from unparasitized crabs and placed in a dish with a crab having a mature Stegophryxus (male removed) whose brood pouch contained late embryos.

July 14. Two cryptonisci have attached to the female Stegophryxus.

July 15. Four cryptonisci now present on the bopyrid. Epicarids are hatching.

July 16. Three cryptonisci remain within the now empty brood pouch. They have developed to the black pigment stage.

July 17. Metamorphosis of cryptonisci continuing; one, at least, has molted.

July 19. The three cryptonisci have entered the juvenile male phase and one is slightly more advanced than the others.

July 24. The three juvenile males are still present and continuing their development.

July 26. One of the juvenile males has disappeared. One of the two remaining ones is permanently removed for examination and drawings are made of it.

August 9. The brood pouch of the female bopyrid is now filled with eggs. (This means that the male has reached maturity.)

August 11. The male was removed for measuring and returned to the brood pouch. Its length is 2.28 mm.

August 24. Development of the eggs has continued normally and today the epicarid larvae are released.

August 26. The male now measures 2.37 mm. Experiment discontinued.

Similar results were obtained when female bopyrids, found in nature with a retinue of cryptonisci present in the brood pouch, were kept under observation. In one case a female Stegophryxus, non-ovigerous and lacking a male, had 18 cryptonisci attached to it. The daily count showed a reduction as follows: 18, 16, 14, 11, 9, 6, 4, 2, 2, 2, 2, 2, 2, 1. The remaining one reached maturity two weeks later and fertilized the eggs of the female which were not released until that time. Another reduction from an initial natural retinue of eight cryptonisci occurred as follows: from 8 to 2 in four days, but these two persisted for 11 more days to become juvenile males, then one disappeared. The survivor became a mature male.

These experiments and observations, while they shed some light on the problem at hand, are inconclusive evidence for or against any theory of the sexual nature of the cryptonisci. They show that cryptonisci that enter the brood pouch of a female metamorphose in the male direction, but what of those that leave early or fail to enter? Could not they be predetermined females unresponsive to masculinizing influences?

II. To settle this point, it was decided to experiment with single cryptonisci. Moreover, only cryptonisci found clinging to the posterior pleopod of a normal crab were used. Nine experiments were undertaken. In five of these the cryptoniscus selected for insertion in the brood pouch of a female was in the brown chromatophore stage; the four other cryptonisci were in the more advanced black stage.

Each of the five Stage 1 cryptonisci remained in the brood pouch and made no efforts to crawl out. One was removed after six days, one after eight, one after eleven, and two after twelve days. Each one had metamorphosed into a male, whose size and extent of development was proportional to the length of time spent in the brood pouch. Those that had been on the female for eleven or twelve days had reached a size of from 1.5 mm. to 1.8 mm.

The experiments with Stage 2 cryptonisci gave different results. In three cases the cryptoniscus crawled out of the brood pouch within a day or two and was either lost or found clinging to the crab instead. One experiment yielded positive results. This cryptoniscus refused repeatedly to attach to the female, but after each escape it was returned to the brood pouch. Finally it remained there, and, eventually, 28 days later, had become a 2 mm. male.

The positive results obtained with the five Stage 1 cryptonisci strongly indicate that cryptonisci that would ordinarily become females can readily be transformed into males through attachment to the body of the female bopyrid, provided the transfer is effected at an early age. Even the one success with a Stage 2 cryptoniscus confirms this. It must be concluded that after a certain period of parasitism on the crab the cryptoniscus becomes female-determined and the direction of its sex development can no longer be changed under ordinary conditions. Subsequent experiments, using juvenile females for transfer, instead of cryptonisci, support this view and will now be briefly recounted.

III. Four attempts were made to transform juvenile females into males. All were completely unsuccessful. Juvenile females in the early post-cryptoniscid phase were used, before they had developed far enough to leave the pleopod of the host for permanent attachment on the abdomen.

One female remained inside the brood pouch for one day, crawled to the exterior and remained there one day, then disappeared.

One left the brood pouch the day after transfer and attached to the abdomen of the crab where it remained for eight days, when the crab died.

One left the brood pouch on the second day and attached to the underside of the telson of the crab. It remained there until the experiment was discontinued 12 days later, and grew from an initial size of .85 mm. to 1.4 mm.

The fourth one was transferred to the brood pouch together with the pleopod to which it was attached. This female remained for five days, then disappeared and could not be recovered for examination.

It would seem that juvenile females are averse to becoming ectoparasites of other more mature females. They leave such an unnatural situation to return to direct parasitism on the crab.[2] There is no evidence that any of the four were modified by their brief sojourn in the brood pouch of another female.

[2] Reverberi (1947) came to the same conclusion with regard to Ione. However, he then placed two females together *in vitro* apart from the host, one being a juvenile female and the other an adult from which the juvenile would have to derive its nourishment. As often as the adult died, another of the same age would be substituted. By this ingenious method he was able to maintain a direct association between a juvenile female and an adult Ione for several months. One case of definite sexual inversion resulted from many trials of this sort. This particular juvenile female underwent external changes and gradually took on the appearance of a male. When killed and sectioned after nearly four months under these experimental conditions, the individual was found to have normal testes partially filled with sperm.

Attempts to Transform Presumptive Males into Females

If presumptive female cryptonisci can be turned into males by altering the environment, the question naturally arises regarding the possibility of producing females from presumptive male cryptonisci. The method of experimentation would be to remove cryptonisci from the brood pouch of a female bopyrid and transfer them to a crab instead. This is the type of experiment on which Caullery pinned his hopes of verifying the theory of sex determination in Epicaridea based upon the type of association with the host.

When this was tried it invariably led to failure because the transferred cryptonisci did not remain attached to the crab long enough to show either positive or negative results. This failure to remain attached need not necessarily be attributed to aversion on the part of the cryptonisci for a strange environment. Indeed, the hazards in the case of direct parasitism on the crab are great. When it is recalled that approximately 10 per cent of the normal crabs have cryptonisci on their surface and only 1.5 per cent of all crabs are infested with female Stegophryxi, it becomes clear that many potential parasites are eliminated through environmental difficulties. Moreover, no success was achieved in numerous attempts to rear to the juvenile female stage cryptonisci found naturally attached to crabs. When crabs bearing cryptonisci are isolated in a dish and examined after a day or two, one finds that the cryptonisci have disappeared. Apparently they are eaten by the crab, since cryptonisci kept in dishes without crabs will remain alive for as long as two weeks. It may be remarked in passing that although isolated cryptonisci survive, they do not develop, nor pass from the brown to the black phase. It is significant that the juvenile female Stegophryxi naturally occurring on crabs can be reared without difficulty in the laboratory. They are actually fixed to the crab and have lost their ability to swim, whereas the cryptonisci, as explained before, are still active and only perch on the crab without fastening themselves to it. Should they disengage themselves even momentarily, they are in danger of being caught up by the currents passing through the gill chambers of the crab and swept in the direction of the crab's mouth. The mouth parts of the crab are in constant motion and any particle that comes in contact with them is trapped and masticated.

The failure of these experiments with cryptonisci removed from the brood pouch of a female and transfered to a crab may, therefore, with considerable assurance, be laid to experimental difficulties.[8] When the proper technique is worked out for Stegophryxus, which will eliminate the hazards facing cryptonisci that attach to crabs, we feel confident that presumptive male cryptonisci can be transformed into females.

[8] This is especially reasonable in view of the fact that Reverberi and Pitotti (1942) experienced a similar lack of success when they tried to implant the cryptoniscus larvae of Ione on the gills of Callianassa. The cryptonisci invariably failed to remain on the host (pp. 148–149). But when they used post-cryptoniscid stages they were successful in bringing about the transformation of juvenile males into females. Ione, unlike Stegophryxus, is a branchial parasite. When juvenile males of Ione, removed from adult females, were placed in the branchial cavity of the host, they soon attached to the branchiae, began to feed, and in the majority of cases remained there more or less permanently. Such males gradually became females.

The Fate of Supernumerary Males and Females

The first series of experiments reported above furnish evidence that although any number of cryptonisci may attach to the same female and develop into juvenile males, only one male is allowed to reach maturity. This point has not been realized by most previous investigators except Caullery (1941) and Reverberi and Pitotti (1942), and hence, in the older literature, several cases of supernumerary males associated with one female bopyrid are mentioned, with the inference that they are highly unusual or evidence of polyandry. Pérez (1924), for example, reports three instances of this from his own observations involving *Pleurocrypta porcellanae* Hesse, *P. galatheae* Hesse, and *Athelges lorifera* Hesse. In the light of recent work, these must be interpreted as cases of extra males in process of development before they have realized the full adult state, since all cases, when analyzed, resolve themselves into the stegophryxoid pattern, namely, one adult functional male accompanied by one or more smaller juvenile males or cryptonisci.

It must be concluded, therefore, that it is a general rule in the bopyrids that only one functional male is permitted at a time. Other potential males, temporarily tolerated, are expelled sooner or later. Whether the legitimate male, by virtue of its larger size, actually drives off the others, or whether they are repelled in some more refined manner is a question still to be answered.

Our observations on the question of excess females show that they, like the supernumerary males, are eliminated sooner or later, usually as juveniles. Only one adult female is ever found on an individual host.

One hermit crab, collected August 23, 1946, carried a large ovigerous Stegophryxus and had in addition four juvenile females in various stages of development attached to the abdomen and pleopods. This crab was isolated for daily observations and in less than a week's time the four juvenile females had disappeared. Two other cases of parasitized crabs, each with a juvenile female present in addition to the adult female Stegophryxus, were observed under laboratory conditions. One juvenile persisted from July 24 to August 10 and grew considerably in size before it was eliminated; the other lasted from July 29 to August 11.

It is significant that the lost juvenile females could not be found in the dish in which the crab had been isolated. Perhaps they drop off and are eaten by the crab. It is more probable, however, that they are driven off by the mature male. One finds, on occasion, the mature male wandering about on the outside of the female brood pouch, and it is not unlikely that the male engages in occasional forays over the abdomen of the crab and drives off or destroys the excess females before they reach maturity.

Histological Observations [4]

Four cryptonisci and ten males, the latter selected to form a graded series of sizes ranging from 1.2 mm. to 2.7 mm., were sectioned and studied histologically to determine the sexual nature of the larvae and the organogenesis of the male gonads.

[4] The author gratefully acknowledges the assistance of Cornelius Sharbaugh, T.O.R., who, under our direction, prepared the slides and made the morphological studies on which this portion of the paper is based.

It was found that males of 2 mm.-length and over could be termed "adult" as judged by the length and development of the testes and the presence of spermatozoa in the vasa deferentia. In such males the reproductive organs are seen as a pair of long tubular masses, beginning in the first thoracic segment, and extending back into the seventh thoracic segment. The testes lie dorso-laterally adjoining the two liver tubes, one on each side of the animal, except for the anterior extremity of each, which occupies a ventro-lateral position in relation to the liver. In the sixth and seventh thoracic segments, the testes become vasa deferentia which open separately to the outside on the ventral surface of the seventh thoracic segment. The beginning of the vas deferens is often dilated to act as a temporary seminal vesicle.

The cells that make up the testis in the anterior-most portion of the organ are all of one type and equally distributed throughout the cross section. Elsewhere, spermatocytes, spermatids, and spermatozoa may be seen arranged in three distinct zones: spermatocytes in the inner zone next to the liver, spermatids in the middle, and spermatozoa in the outer zone (Fig. 4). The cells of the inner and middle zones are grouped into areas or patches, but those of the outer zone extend without interruption the entire length of the testis.

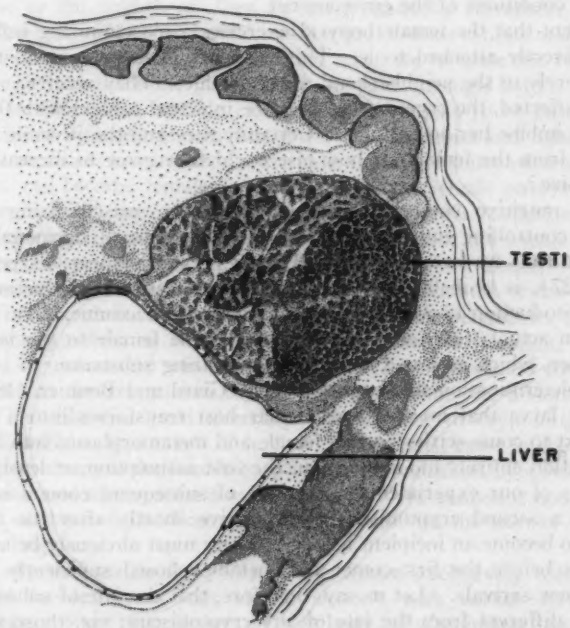

FIGURE 4. Right half of transverse section through the third thoracic segment of a male *Stegophryxus hyptius* measuring 2.7 mm. in length. The testis, dorso-lateral to the liver, shows the characteristic zonal arrangement: spermatocytes in the inner zone closest to the liver, spermatids intermediate in position, and spermatozoa in the outer zone. × 400.

Males of approximately 1.5 mm.-length are essentially similar to the larger males except that the testes are shorter, beginning in the second or third thoracic segment, and the three characteristic zones appear only in the posterior portion. Anteriorly, the testes contain spermatocytes and spermatids, but no spermatozoa.

In the smallest male examined, length 1.2 mm., the testis on the left side was undeveloped; that on the right was short and contained spermatocytes and spermatids only. These occupied a relatively short middle section, with anterior and posterior extremities appearing empty.

None of the four cryptonisci examined, three in cross section and one in longitudinal section, showed either gonads or groups of cells that might be regarded as traces of gonads.

Discussion and Conclusions

The experiments reported on here seem to indicate that the cryptoniscus larvae of Stegophryxus can develop in either of two directions: into males under conditions of parasitism on a female bopyrid, or into females, under conditions of parasitism on a hermit crab. As in the case of Bonellia (Baltzer, 1914), the larvae are apparently indifferent, with both sex potencies. Which potency will be realized depends on the conditions of the environment.

It is evident that the female bopyrid exercises a masculinizing influence on the cryptonisci directly attached to it. This influence does not extend to cryptonisci which are merely in the neighborhood of the female. They receive no male stimulus. To be affected, the cryptoniscus must be in actual contact with the female and perhaps even imbibe her body fluids. Certainly they and the juvenile males receive nourishment from the female, or how else could they grow to increase as much as threefold in size?

Whether nutritive conditions alone provide the masculinizing stimulus, or whether the controlling influence is a specific substance of hormonal nature, is a question requiring further experimental study. Nourishment, as pointed out by Zimmer (1927), is probably the determining factor in the production of females, but for the production of males it seems necessary to assume, as in Bonellia, the transfer of an actual secretion from the body of the female to the larvae that are attached to her, which acts as a specific masculinizing substance.

The sex-determination theory proposed by Giard and Bonnier (1887), namely, that the first larva that invades a particular host transforms into a large female, while the next to come settles on this female and metamorphoses into a dwarf male, is an explanation entirely too simple; but the first assumption, at least, is supported by the results of our experiments. The fate of subsequent comers is less certain. Conceivably, a second cryptoniscus might arrive shortly after the first and also settle down to become an incipient female. There must obviously be a time interval of some days before the first-comer has metamorphosed sufficiently to invite the attention of new arrivals. Let us say, therefore, that the fate of subsequent comers is in no way different from the fate of any cryptoniscus; viz. those that settle directly on a crab become female-determined, while those that settle on a female of their species become male-determined.

One of the objections to the theory of Giard and Bonnier has been the fact that two females are sometimes found on the same host. Thus Hiraiwa (1936) says:

"If the female in the (branchial) cavity makes the later invader into male, why are two females found in one and the same cavity?"

The answer to this objection is now clear. A female does not influence the sex of later invaders unless they settle directly on her body. Should a cryptoniscus settle on the crab, no matter how close in position to a large female, the cryptoniscus will not be affected by this proximity so far as its sex-determination is concerned.

The sex determination theory of Smith (1909) and Goldschmidt (1920) with reference to the Bopyridae requires no discussion. It is eliminated by the facts presented in describing the life cycle of Stegophryxus and has previously been sufficiently criticized by Hiraiwa (1936).

Hiraiwa's own theory, disclaiming as it does differentiation due to environmental factors, is not in harmony with the results of the experiments reported here. His assumption, however, that the free-swimming larvae are not males but are sexually undifferentiated is in agreement with our findings.

Caullery's theory (1941) finds ample confirmation in the results of our experiments. Although the exact experimental verification he hoped for has not yet been realized by us, the converse experiment of transferring cryptonisci from the host to the female bopyrid has yielded satisfactory evidence that the sexes are not fixed from the start.

Coming now to the studies on *Ione thoracica* made by Reverberi and Pitotti (1942) and Reverberi (1947), and their relation to the observations and deductions reported here on *Stegophryxus hyptius*, we find when we tabulate the two for comparison (Table 1) that the same general pattern runs through both. Some, perhaps most, of the differences that do exist are modifications to be expected when comparing species of different genera that differ also in habits and habitat. Thus, since Ione is a branchial parasite, the larvae have the opportunity of settling on the gills of the host (to become female-determined), on the female parasite itself (to become male-determined), or on the skin of the host (likewise to become male-determined). Stegophryxus presents a simpler condition since the female is attached not to the branchiostegite but to the abdomen of the host. Therefore only two substrates are selected for attachment by the larvae: the abdomen of the host or the female herself. If abundant nourishment is the factor that determines females and less abundant nourishment, as Reverberi and Pitotti at first thought, determines males, it is hard to understand why the abdomen of Pagurus should furnish abundant nourishment to the cryptonisci of Stegophryxus while the abdomen of Callianassa should not likewise yield abundant nourishment to cryptonisci of Ione. Later (1947), Reverberi, as a result of further experiments, came to the conclusion that the larvae that attach to the female parasite are masculinized by a sex-determining substance produced by the female rather than by "less abundant nourishment"; but the problem of the so-called "complementary males" on the skin of Callianassa is still very puzzling.

Reverberi's experiments on Ione were made almost exclusively with the older post-cryptoniscid larvae; ours dealt almost entirely with earlier larvae in the cryptoniscus stage. Since the larvae on the body of Callianassa were already presumptive males, his chief experiments were to make females out of them. In Stegophryxus, on the other hand, the cryptonisci on the body of the host are presumptive females, hence our main experiments were to make males out of them. All in all, the two studies complement and illuminate each other. Together they

TABLE 1

A comparison between Ione thoracica and Stegophryxus hyptius with respect to sex-determination. Data for Ione compiled from the papers of Reverberi and Pitotti (1942) and Reverberi (1947)

Ione thoracica	*Stegophryxus hyptius*
1. Adult female lives in the branchial cavity of Callianassa.	1. Adult female lives on the abdomen of Pagurus.
2. Females result from cryptonisci that settle on the gill of the host.	2. Females result from cryptonisci that settle on the abdomen of the host.
3. Females secrete a diffusible substance capable of attracting free cryptoniscus larvae.	3. Same.
4. Cryptonisci that attach to the female become males.	4. Same.
5. Only one adult male is retained.	5. Same.
6. Cryptonisci that attach to the skin of the host became complementary males, capable of replacing the lost male of an adult pair.	6. Cryptonisci that attach to the skin of the host become juvenile females. Replacement of lost males occurs from free cryptonisci only.
7. Juvenile males, removed from adult females, can be transformed into females by placing them on the gills of the host.	7. No evidence that juvenile males can undergo sex reversal, but presumptive male cryptonisci could probably be transformed into females if they left the female and obtained nourishment from the host instead.
8. A widowed male can become female if it succeeds in attaching to the host gill and getting abundant nourishment.	8. It is unlikely that males, once differentiated, could undergo sex reversal.
9. The few comparable experiments reported did not yield results.	9. Presumptive female cryptonisci become males when removed from the host and placed on a female parasite.
10. Evidence supplied in 1947 that differentiated females can undergo sex reversal.	10. No evidence that differentiated females have the capacity for reversal to male.
11. Larvae that engorge host blood directly become females if nourishment is abundant (taken from gills), but become complementary males if nourishment is less abundant (taken from skin of host).	11. All larvae in a position to feed on the host directly become females. Only larvae that attach to the female become males.
12. Reverberi first attributed maleness, when larvae are attached to a female, to "less abundant food," but later (1947) explained it as due to masculinizing substance.	12. Data favora hypothesis of a masculinizing substance produced by the female.
13. Gonad tissue first appears in older post-cryptoniscid forms and the earliest gonad is indifferent.	13. Cryptonisci lack gonads. The earliest gonad in juvenile males is a testis. No data presented on the earliest type of gonad in a juvenile female.
14. Sex-determination is environmental, comparable (with modifications) to that described for Ophryotrocha.	14. Sex-determination is environmental, comparable (with modifications) to that described for Bonellia.

fulfill the expectations of Caullery and reveal a fundamental plan of sex-determination and sex-differentiation, a plan, however, that can be expected to exhibit minor variations when utilized by different genera of Bopyridae.

SUMMARY

Stegophryxus hyptius Thompson, an ectoparasite on the abdomen of the hermit crab *Pagurus longicarpus* Say, seeks the definitive host as a cryptoniscus larva.

The free-swimming cryptonisci are sexually undifferentiated and sexually undetermined. Differentiation follows fixation and is dependent on environmental factors. These conclusions are justified on the basis of the following observations and experiments.

1. Cryptonisci that settle directly on the host develop into females; those that attach to a female bopyrid develop into males.

2. Changes in the color pattern of the cryptonisci following fixation furnish criteria for judging the length of time they have been subjected to a particular environment.

3. Histological examination of the cryptoniscus shows that the gonads are not yet present as recognizable structures.

4. Presumptive female cryptonisci, if removed from the host at an early age and transferred to the brood pouch of a female Stegophryxus, will transform into males.

5. The failure of the converse experiment involving transfer of presumptive male cryptonisci from the female parasite to the host can definitely be laid to experimental difficulties.

6. The factor that determines maleness is a masculinizing substance imbibed with food from the female, but this substance does not act at a distance.

7. Attachment of supernumerary females as well as excess differentiating males is terminated sooner or later so that a particular crab is host to only a single adult female paired with one functional male.

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STUDIES ON MARINE BRYOZOA, III. WOODS HOLE REGION BRYOZOA ASSOCIATED WITH ALGAE

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Introduction

Heretofore there has been no extensive study made of the association between bryozoa and algae, except for the studies by Joliet (1877) and Prenant and Teissier (1924, 1927, 1932), although incidental association notes are scattered throughout taxonomic papers.

The purposes of the present study are several: (1) to note any association that may exist between algae and certain bryozoa (bryozoa likely to be encountered in the Woods Hole region); (2) to aid collectors of bryozoa, since in some instances by collecting specified algae one is almost sure to find a number of desired bryozoa; (3) to make any observations possible on the tentacle number, the occurrence of embryos, larvae and ovicells in specimens collected during the summer months, and (4) to report any additional species from the collection area.

Collection Data

The materials used in this study were marine algae of three general groups: (1) freshly collected specimens; (2) dry, pressed specimens mounted at various times in the past on herbarium sheets; and (3) specimens preserved in the Botany Course Stock Collection of the Marine Biological Laboratory (M. B. L.) of Woods Hole, Mass. More species were examined than are here recorded but only those 37 algal species which had bryozoa growing on them are here listed.

Some of the fresh material was obtained in the intertidal zone by shore collecting; some had drifted in from deeper waters some distance from shore; and some had been dredged with a scallop dredge from waters about 20 to 60 feet in depth.

Some of the material was collected by the authors, some by the M. B. L. Invertebrate Zoology and Botany classes on their field trips, some by the M. B. L. Supply Department and Collecting Crew, and one algal species by Dr. Maxwell Doty of Northwestern University. To all these the authors wish to express their most sincere appreciation, and especially to Dr. William Randolph Taylor of the University of Michigan for very helpful suggestions, continued kindly interest, and for specimens of *Membranipora tuberculata* and their algal hosts from his own collection.

The *Laminaria longicruris* was brought in by Dr. Doty from Race Point, near Provincetown, Mass. on VIII–18–1947. The Rye Ledge, Rye, New Hampshire specimens of *Chondrus crispus, Phycodrys rubens* and *Phyllophora membranifolia* were collected by the junior author on IX–30–1945. The New Rochelle, New York specimens of *Ascophyllum nodosum, Ascophyllum Mackaii, Chondrus crispus* and *Laminaria Agardhii* were observed on IX–16 and 22–1945, and on X–3–1946 by the senior author. The remainder of the specimens were collected in the waters around and between North Falmouth, Martha's Vineyard, Woods Hole, Vineyard Sound, Buzzards Bay, Penikese Island and New Bedford, all in Massachusetts, during the summer months, between June 30 and August 31 approximately, over a period of several years (Sept. 1874, 1916, 1930, 1935, 1936, 1938, 1939, 1944 through 1947). Specimens from the five earliest years came from the M. B. L. Botanical Collection, some from wet mounts and some from dried herbarium mounts. Exact records, dates and collection numbers of all these algal specimens are on file, but only a very condensed amount of these data is included in Tables I to IV, to save space.

The senior author identified the bryozoa, the junior author classified the algae and collected many of them.

TABLE I

Collection sites and bryozoa associated with green algae

	Cladophora gracilis f. tenuis	*Enteromorpha intestinalis*	*Ulva Lactuca* var. *rigida*	Total No. green algae having this bryozoan species
Bowerbankia gracilis		x	x	2
Bugula turrita	x	x	x	3
Cryptosula pallasiana		x	x	2
Flustrella hispida			x	1
Pedicellina cernua	x			1
Total No. bryozoan spp. on this alga	2	3	4	
Collected at Woods Hole, Mass.	x	x	x	

To date there have been reported 84 species of marine bryozoa from the Woods Hole region, by Osburn (1912) mostly, and by Rogick (1945a, 1948). Some of the Woods Hole species have been previously reported from such widely separated regions as the coast of Africa, Australia, the Azores, Brazil, Denmark, Great Britain, Japan, New Zealand, Panama, Zanzibar, and the Pacific coast of North America. These were reported from various substrates as shells, rocks, hydroids, algae, various animals, piles, and other submerged objects. The present study was mainly directed toward finding the exact algal species on which bryozoa grow. Previous reports from the Woods Hole region occasionally did indicate the algal genera but rarely the species on which the bryozoa occurred. The following lists of algae and bryozoa are of species collected or examined for the present paper.

Below follows a list of 37 algae on which were commonly found various bryozoans. Taylor's (1937) classification is used.

TABLE II

Collection sites and bryozoa associated with brown algae

	Ascophyllum Mackaii	*Ascophyllum nodosum*	*Chorda Filum*	*Cladostephus verticillatus*	*Dermarestia aculeata*	*Fucus evanescens*	*Fucus vesiculosus*	*F. vesiculosus spiralis*	*Laminaria Agardhii*	*Laminaria longicruris*	*Sargassum Filipendula*	Total No. brown algal spp. associated with this bryozoan
Aetea sica		x	x	x			x	x	x		x	7
Aeverrillia armata									x			1
Aeverrillia setigera		x							x		x	3
Alcyonidium polyoum									x	x		2
Bowerbankia gracilis		x	x	x			x	x			x	6
Bowerbankia imbricata							x		x		x	3
Bugula cucullifera						x				x		2
Bugula flabellata							x					1
Bugula turrita		x	x				x	x	x		x	6
Callopora aurita									x			1
Cribrilina punctata									x			1
Crisia eburnea		x			x				x		x	4
Cryptosula pallasiana	x	x						x	x			4
Electra hastingsae								x	x			2
Electra pilosa		x			x	x		x	x	x	x	7
Flustrella hispida		x					x					2
Hippothoa hyalina		x						x	x		x	4
Membranipora lacroixii(?)	x											1
Microporella ciliata									x			1
Pedicellina cernua		x					x		x			3
Schizoporella biaperta					x				x			2
Schizoporella unicornis								x	x			2
Scruparia ambigua					x	x			x			3
Scruparia clavata									x			1
Smittina trispinosa									x			1
Total No. bryozoan spp. found on this alga	2	10	3	2	4	3	7	8	20	3	8	
Collected at Woods Hole		x	x	x			x	x	x		x	
Collected at North Falmouth		x						x			x	
Collected in Vineyard Sound			x		x	x					x	
Collected at Martha's Vineyard					x	x	x		x			
Collected at Penikese Island								x				
Collected at Provincetown										x		
Collected at New Rochelle	x	x							x			

TABLE III

Collection sites and bryozoa associated with red algae

	Agardhiella tenera	*Callithamnion roseum*	*Ceramium rubriforme*	*Ceramium rubrum*	*Champia parvula*	*Chondrus crispus*	*Corallina officinalis*	*Cryptopleura* sp.	*Cystoclonium purpureum cirrhosum*	*Gracilaria confervoides*	*Gracilaria foliifera*	*Lithothamnium* sp.	*Phycodrys rubens*	*Phyllophora Brodiaei*	*Phyllophora membranifolia*	*Plumaria elegans*	*Polysiphonia elongata*	*Polysiphonia nigra*	*Polysiphonia nigrescens*	*Polysiphonia violacea*	*Rhodomela subfusca*	*Rhodymenia palmata*	Total No. red algal spp. associated with this bryozoan
Aetea sica				x		x			x	x		x	x	x	x				x	x	x		11
Aeverrillia armata															x								1
Aeverrillia setigera					x	x									x				x		x		5
Alcyonidium polyoum						x								x	x								3
Bowerbankia gracilis	x			x	x	x	x		x		x			x	x				x				10
Bowerbankia imbricata						x					x												2
Bugula cucullifera															x							x	2
Bugula turrita						x			x				x	x	x				x				7
Callopora aurita													x		x								2
Cellepora dichotoma						x				x				x	x								4
Cribrilina annulata													x										1
Cribrilina punctata						x							x	x	x					x		x	6
Crisia eburnea				x	x	x	x		x	x			x	x	x	x		x	x	x		x	14
Cryptosula pallasiana						x			x					x	x					x			5
Electra pilosa						x			x				x	x	x	x		x		x	x	x	10
Flustrella hispida						x									x								2
Hippoporina contracta														x	x								2
Hippothoa hyalina				x		x	x		x	x			x	x	x		x			x	x	x	13
Lichenopora hispida													x		x								2
Membranipora lacroixii(?)						x									x								2

TABLE III—*Continued*

	Agardhiella tenera	*Callithamnion roseum*	*Ceramium rubriforme*	*Ceramium rubrum*	*Champia parvula*	*Chondrus crispus*	*Corallina officinalis*	*Cryptopleura* sp.	*Cystoclonium purpureum cirrhosum*	*Gracilaria confervoides*	*Gracilaria foliifera*	*Lithothamnium* sp.	*Phycodrys rubens*	*Phyllophora Brodiaei*	*Phyllophora membranifolia*	*Plumaria elegans*	*Polyides rotundus*	*Polysiphonia elongata*	*Polysiphonia nigra*	*Polysiphonia nigrescens*	*Polysiphonia variegata*	*Rhodomela subfusca*	*Rhodymenia palmata*	Total No. red algal spp. associated with this bryozoan
Membranipora tuberculata								x																1
Microporella ciliata						x								x	x								x	4
Pedicellina cernua						x			x		x			x	x					x	x			7
Schizoporella biaperta						x	x		x				x	x	x					x				7
Schizoporella unicornis						x			x						x								x	4
Scruparia ambigua		x	x	x		x			x							x			x		x			8
Smittina trispinosa						x								x	x									3
Total No. bryozoan spp. on this alga	1	1	1	5	3	20	4	1	11	4	3	1	10	15	23	3	2	1	3	7	8	4	7	
Collected at Woods Hole	x			x	x	x				x	x	x		x	x					x	x	x		
Collected at Martha's Vineyard		x	x	x		x	x		x				x	x		x	x	x	x		x		x	
Collected in Vineyard Sound				x											x								x	
Collected at North Falmouth						x			x											x		x		
Collected on Penikese Island						x																		
Collected at New Bedford	x														x					x				
Collected at New Rochelle						x																		
Collected at Rye, N. H.						x							x		x									
From Puerto de la Paloma, Uruguay*								x																

* Specimens sent by Dr. W. R. Taylor.

TABLE IV

Collection sites and other Bryozoan data

	Penikese Island, Mass.	New Bedford, Mass.	North Falmouth, Mass.	Vineyard Sound, Mass.	Martha's Vineyard, Mass.	Woods Hole, Mass.	New Rochelle, New York	Rye, New Hampshire	Provincetown, Mass.	Puerto de la Paloma, Uruguay	No. of algal spp. from which bryozoan was reported in Tables I, II, III	Partial list of the No. of algal spp. or genera from which this bryozoan was reported by other workers. See under each bryozoan species discussion	Tentacle number. Personal observations unstarred, others starred	In filled-ovicell, embryo-producing or larva-releasing stage at Woods Hole
1. *Aetea sica*			x	x	x	x					18		9–11	July 30–Aug. 6±
2. *Aeverrillia armata*		x			x						2		8	
3. *Aeverrillia setigera*		x				x					8		8	
4. *Alcyonidium polyoum*		x				x	x		x		5	5	16, 20*	
5. *Bowerbankia gracilis*		x	x	x	x	x					18		8	
6. *Bowerbankia imbricata*					x	x	x				5	6	10	All of Aug.±
7. *Bugula cucullifera*		x		x					x		4		13	Aug. 1–18 ±
8. *Bugula flabellata*					x						1			June 1–Nov. 15
9. *Bugula turrita*			x	x	x	x					16		14	June 30–Aug. 15±
10. *Callopora aurita*					x	x		x			3			
11. *Cellepora dichotoma*					x	x					4			July 25±
12. *Cribrilina annulata*								x			1	1		
13. *Cribrilina punctata*	x				x	x		x			7			
14. *Crisia eburnea*		x	x	x	x	x		x			18	2	8*	Aug. 8±
15. *Cryptosula pallasiana*			x		x	x	x				11	4	16	June–Sept.±

TABLE IV—*Continued*

	Penikese Island, Mass.	New Bedford, Mass.	North Falmouth, Mass.	Vineyard Sound, Mass.	Martha's Vineyard, Mass.	Woods Hole, Mass.	New Rochelle, New York	Rye, New Hampshire	Provincetown, Mass.	Puerto de la Paloma, Uruguay	No. of algal spp. from which bryozoan was reported in Tables I, II, III	Partial list of the No. of algal spp. or genera from which this bryozoan was reported by other workers. See under each bryozoan species discussion	Tentacle number. Personal observations unstarred, others starred	In filled-ovicell, embryo-producing or larva-releasing stage at Woods Hole
16. *Electra hastingsae*						x	x				2	1		
17. *Electra pilosa*	x	x	x	x	x	x	x	x	x		17	8	12–14	
18. *Flustrella hispida*						x					5	3	13	
19. *Hippoporina contracta*						x					2		12*	
20. *Hippothoa hyalina*	x	x	x	x	x	x		x			17	5	12*–14*	July–Aug.±
21. *Lichenopora hispida*								x			2			
22. *Membranipora lacroixii*(?)							x				3		11	
23. *Membranipora tuberculata*										x	1	3		
24. *Microporella ciliata*					x	x					5	3	13*–14*	Aug.±
25. *Pedicellina cernua*		x	x	x	x	x	x				11	6	8–12; 8*–24*	
26. *Schizoporella biaperta*			x		x	x					9		12	July–Aug.±
27. *Schizoporella unicornis*		x	x		x	x					6	3	about 18*	July–Aug.±
28. *Scruparia ambigua*			x		x						11		10	
29. *Scruparia clavata*					x						1			
30. *Smittina trispinosa*			x		x	x					4			

* The references for the starred* numbers in the Tentacle No. column can be found under each species' descriptive section.

± The plus and minus signs in the last column mean that embryos or larvae very likely were present earlier or later than the days and months listed in the last column.

List of Collected Algae

Chlorophyceae (green algae)

1. *Cladophora gracilis* (Griffiths) Kützing, forma *tenuis* Farlow
2. *Enteromorpha intestinalis* (Linnaeus) Link (a proliferous form)
3. *Ulva Lactuca* Linnaeus var. *rigida* (C. Agardh) LeJolis

Phaeophyceae (brown algae)

4. *Ascophyllum Mackaii* (Turner) Holmes et Batters
5. *Ascophyllum nodosum* (Linnaeus) LeJolis
6. *Chorda Filum* (Linnaeus) Lamouroux
7. *Cladostephus verticillatus* (Lightfoot) C. Agardh
8. *Desmarestia aculeata* (Linnaeus) Lamouroux
9. *Fucus evanescens* C. Agardh
10. *Fucus vesiculosus* Linnaeus
11. *Fucus vesiculosus* var. *spiralis* Farlow
12. *Laminaria Agardhii* Kjellmann
13. *Laminaria longicruris* De la Pylaie
14. *Sargassum Filipendula* C. Agardh

Rhodophyceae (red algae)

15. *Agardhiella tenera* (J. Agardh) Schmitz
16. *Callithamnion roseum* (Roth) Harvey
17. *Ceramium rubriforme* Kylin
18. *Ceramium rubrum* (Hudson) C. Agardh
19. *Champia parvula* (C. Agardh) Harvey
20. *Chondrus crispus* (Linnaeus) Stackhouse
21. *Corallina officinalis* Linnaeus
22. *Cryptopleura* sp.
23. *Cystoclonium purpureum* (Hudson) Batters var. *cirrhosum* Harvey
24. *Gracilaria confervoides* (Linnaeus) Greville
25. *Gracilaria foliifera* (Forsskål) Børgesen
26. *Lithothamnium* sp.
27. *Phycodrys rubens* (Hudson) Batters
28. *Phyllophora Brodiaei* (Turner) J. Agardh
29. *Phyllophora membranifolia* (Goodenough et Woodward) J. Agardh
30. *Plumaria sericea* (Harvey) Ruprecht
31. *Polyides rotundus* (Gmelin) Greville
32. *Polysiphonia elongata* (Hudson) Harvey
33. *Polysiphonia nigra* (Hudson) Batters
34. *Polysiphonia nigrescens* (Hudson) Greville
35. *Polysiphonia variegata* (C. Agardh) Zanardini
36. *Rhodomela subfusca* (Woodward) C. Agardh
37. *Rhodymenia palmata* (Linnaeus) Greville

Below is a list of the 30 bryozoan species which were found growing on the various algae examined by the authors.

List of Collected Bryozoa

ENTOPROCTA

1. *Pedicellina cernua* (Pallas) 1771

ECTOPROCTA

Cyclostomata or Stenolaemata

2. *Crisia eburnea* (Linnaeus) 1758
3. *Lichenopora hispida* (Fleming) 1828

Ctenostomata

4. *Aeverrillia armata* (Verrill) 1873
5. *Aeverrillia setigera* (Hincks) 1887
6. *Alcyonidium polyoum* (Hassall) 1841
7. *Bowerbankia gracilis* Leidy 1855
8. *Bowerbankia imbricata* (Adams)1800
9. *Flustrella hispida* (Fabricius) 1780

Cheilostomata

10. *Aetea sica* (Couch) 1844
11. *Bugula cucullifera* Osburn 1912
12. *Bugula flabellata* (Thompson) 1848
13. *Bugula turrita* (Desor) 1848
14. *Callopora aurita* (Hincks) 1877
15. *Cellepora dichotoma* Hincks 1862
16. *Cribrilina annulata* (Fabricius) 1780
17. *Cribrilina punctata* (Hassall) 1841
18. *Cryptosula pallasiana* (Moll) 1803
19. *Electra hastingsae* Marcus 1938
20. *Electra pilosa* (Linnaeus) 1767
21. *Hippoporina contracta* (Waters) 1899
22. *Hippothoa hyalina* (Linnaeus) 1767
23. *Membranipora lacroixii* (?)
24. *Membranipora tuberculata* (Bosc) 1802
25. *Microporella ciliata* (Pallas) 1766
26. *Schizoporella biaperta* (Michelin) 1842
27. *Schizoporella unicornis* (Johnston) 1847
28. *Scruparia ambigua* (d'Orbigny) 1841
29. *Scruparia clavata* Hincks 1857
30. *Smittina trispinosa* (Johnston) 1825

Bryozoan Growths on Algae

The bryozoa form white, grey, yellow, salmon-pink, or brown growths on the algae. Some bryozoan colonies are thin, flat, encrusting and closely adherent. Others are dendritic, arborescent, or may form a fuzzy mass of tiny vesicles. Still others coat the algae with a gelatinous, rubbery, or leathery film. The calcareous

bryozoa often retain their zooecial patterns and specific characteristics pretty well even though the algal host specimens have been dried and pressed in the normal course of herbarium sheet mounting. It was no harder to identify *Hippothoa hyalina* from a dry 1874 herbarium mount of *Phycodrys rubens* than from a freshly collected alga.

Bryozoa grow on various parts of the algal plant. The holdfast processes of Laminaria and related forms are excellent sites for attachment of at least 21 hard, horny, or soft bryozoan species. Bryozoa grow on and between the holdfast processes as well as on the rocks to which the holdfasts adhere. Laminaria and Rhodymenia blades are favorite attachment sites for *Electra pilosa* which is very common and especially abundant on these algae, sometimes coating both sides of the entire blade for an area of several inches with a thin, frosty-white, single-layered cover of contiguous bryozoan colonies. *Membranipora tuberculata* has the same habit of extensively encasing its algal hosts with the fine bryozoan mesh.

The basal or most proximal parts of *Chondrus crispus* and Phyllophora are encrusted by many bryozoans like Aeverrillia, Bowerbankia, *Cellepora dichotoma* and *Hippothoa hyalina,* while the most distal tips are somewhat less often utilized for bryozoan attachment. Sometimes, if the bryozoan growth is especially rich or dense on these two algal genera, the whole blade may be covered. Alcyonidium may encase a whole blade and sometimes extend even beyond the tips of the plant. The two Schizoporellae also may grow so readily as to produce shelf-like extensions of the colony beyond the plant thallus.

The basal parts of Ascophyllum and Fucus are generally favored by the bryozoan colonies, as are the crevices and depressions around the airbladders and where branches originate. *Flustrella hispida* and Bowerbankia particularly favor these plants.

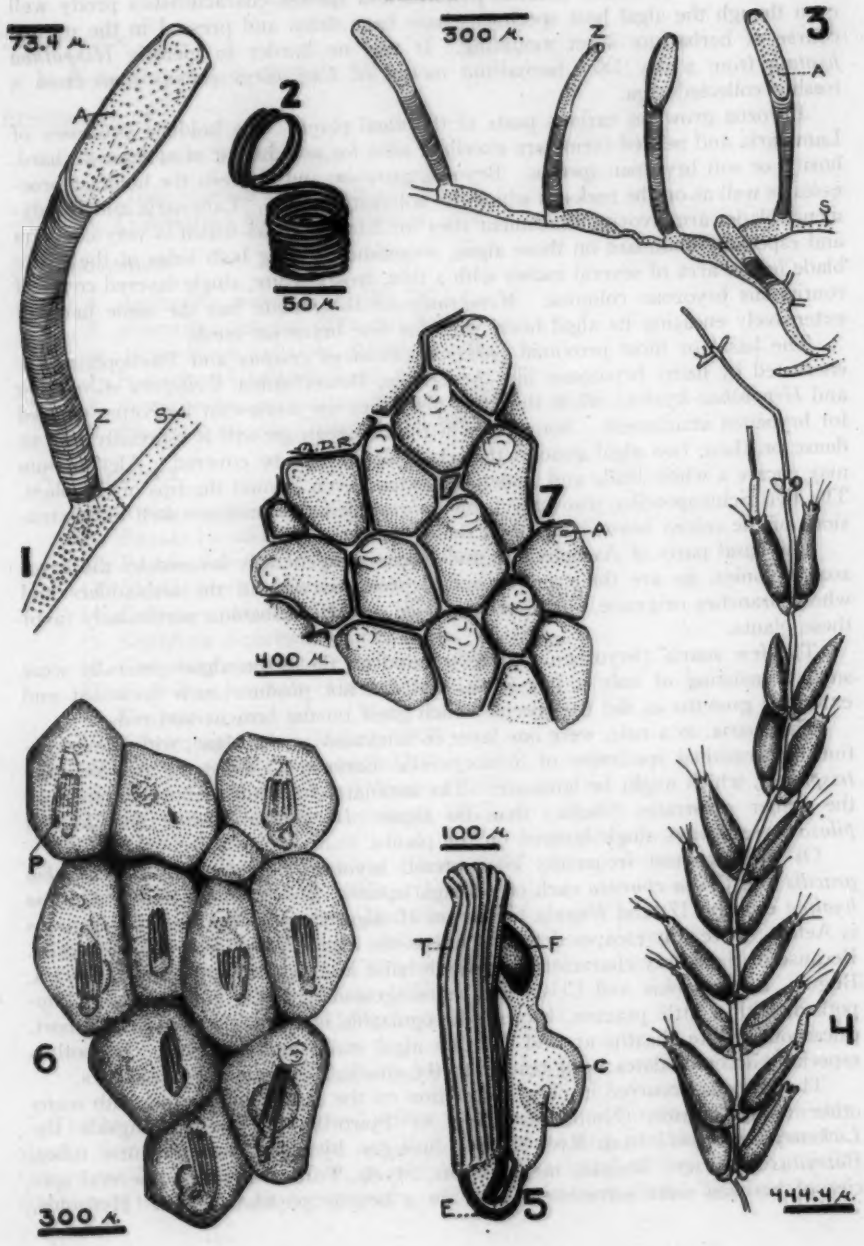
The few zoaria (bryozoan colonies) found on the green algae generally were small, consisting of only a few zoids, and did not produce such luxuriant and extensive growths as did the species which grew on the browns and reds.

The zoaria, as a rule, were one layer in thickness on the algae, with the exception of occasional specimens of *Schizoporella biaperta, S. unicornis* and *Smittina trispinosa,* which might be laminate. The laminate condition is more common on the firmer substrates (rocks) than on algae. *Hippothoa hyalina* and *Electra pilosa* were always single-layered on the plants.

Of the six most frequently encountered bryozoa (*Aetea sica, Bowerbankia gracilis* and *Crisia eburnea* each on 18 algal species, *Electra pilosa* and *Hippothoa hyalina* each on 17, and *Bugula turrita* on 16 algal species) the least conspicuous is Aetea. It readily escapes detection unless the alga is examined microscopically. Because of their very characteristic growth habit and general appearance, Electra, Bugula, Bowerbankia and Crisia can be recognized with the unaided eye. Hippothoa, with a little practice, becomes recognizable because it forms small, short, calcareous, white sheaths around the thin algal stalks and filaments. Hippothoa especially accommodates itself readily to the smallest filaments and branches.

The bryozoa occurred in close association on the same algal thallus with many other animal forms. Numerous shells of Spirorbis sp. grew alongside the *Lichenopora hispida* from Rye, N. H. Sponges, hydroids, annelid worm tubes, *Botryllus schlosseri, Molgula manhattensis,* Styela, Foraminifera, and several species of bryozoa were sometimes found on a heavily populated alga. Hydroids,

PLATE I



Foraminifera, and several bryozoan species often were found on the same blade of Ascophyllum, Chondrus, Laminaria, Phycodrys, or Phyllophora. Aeverrillia and Aetea would sometimes grow on Bugula and hydroid colonies as well as on algal thalli.

The tentacle number and the time of larval production were obtained for some species but not for all because sometimes the colonies died before they could be examined, and sometimes the organisms were so exasperatingly slow in extending their tentacles for a count. Such data as could be obtained are listed in Table IV and also in the species' descriptive section which follows.

Aetea Sica

(Figures 1–3)

Aetea sica is fairly common, although not reported from this region previously. It forms a thin, white, bristly tracery on 18 different algal species. The zoids resemble fine upright tubes just big enough to be seen with the unaided eye. Slender stolons connect the bases of the upright zoids and adhere closely to the substratum (Fig. 3). Nine to eleven tentacles were counted in a few zoids. Ovicells were filled with live developing pinkish larvae from at least July 31 through August 6.

The feature by which Marcus (1937, p. 29) distinguishes *Aetea sica* from the previously reported *Aetea anguina* is the ratio of the aperture (opesium) length to opesium width. The opesial ratio for *A. anguina* is between 1.7 : 1 and 2 : 1. For *A. sica* it is between 2.6 : 1 and 4 : 1. In Figure 1, one zoid has a 4 : 1 ratio. If the ratio is a valid characteristic of the two species, then some of the previously reported *Aetea anguina* from the Woods Hole area must belong to *Aetea sica*.

Plate I *

Figure 1. *Aetea sica.* Upright zoid (Z) growing from a punctate stolon (S) enlargement. The opesium (A) of this zoid is about three times as long as wide (a 3 : 1 ratio). The scale above applies to this figure. Hadley Harbor specimens, VII–28–1939.

Figure 2. *Aetea sica.* Detail of a broken stalk.

Figure 3. *Aetea sica.* A colony of five zoids (Z) arising from stolons (S). The upper right zoid has an opesium (A) about four times longer than wide (4 : 1 opesial ratio). The scale above applies to this figure.

Figure 4. *Aeverrillia armata.* A sprig of a colony collected from Lagoon Pond, Martha's Vineyard, VIII–17–1945.

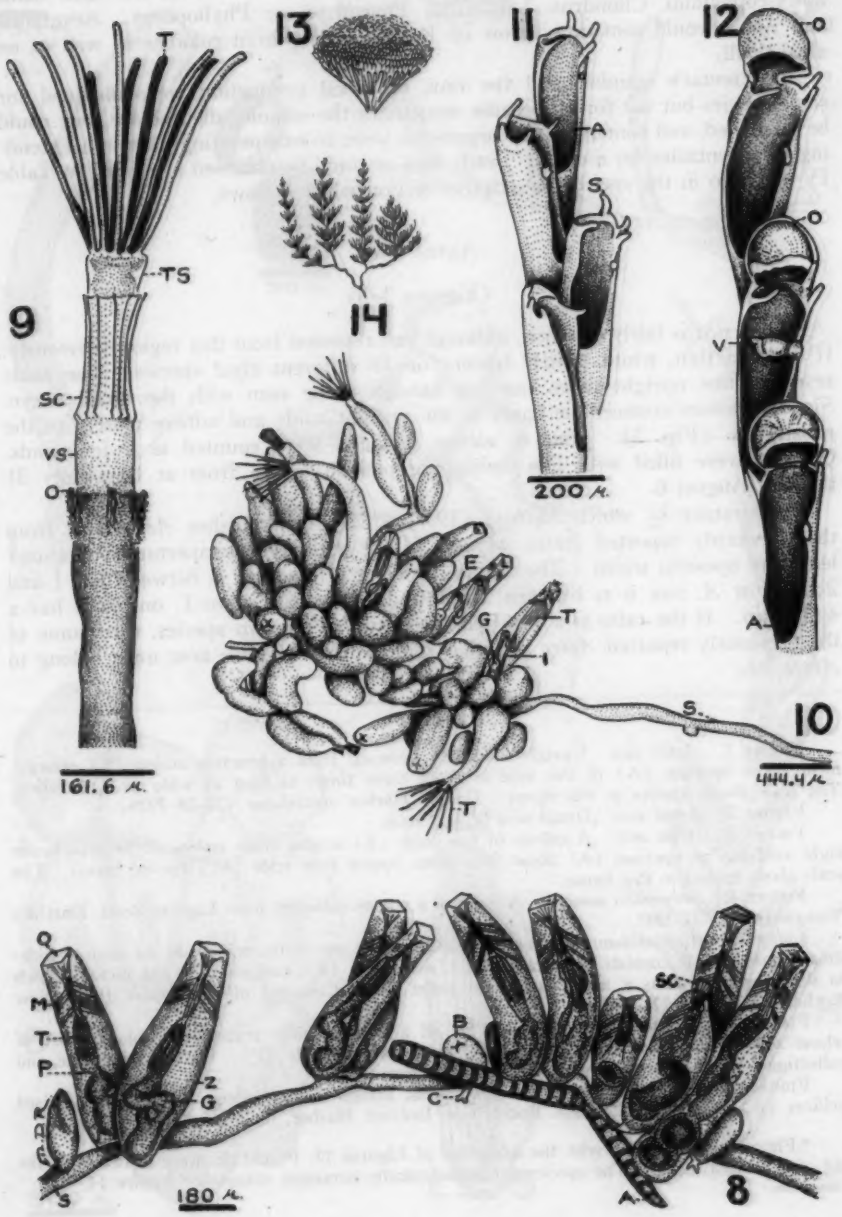
Figure 5. *Alcyonidium polyoum.* A polypide torn out of the colony, in its natural withdrawn position. It consists of tentacles (T), esophagus (E), caecum (C) and rectum which in this sketch contains a large dark fecal pellet (F). Collected off Davenport Park, New Rochelle, N. Y., on IX–22–1945.

Figure 6. *Alcyonidium polyoum.* Part of a young, fairly transparent colony most of whose zooecia contain a sketchily outlined withdrawn polypide (P). Of the same date and collecting locality as specimens of the preceding figure.

Figure 7. *Alcyonidium polyoum.* Somewhat thicker-walled colony, with slightly raised orifices (A). Collected at Black Rock, New Bedford Harbor, on VIII–8–1945.

* Figures on all plates, with the exception of Figures 13, 14 and 28, were drawn with the aid of a camera lucida. The species are alphabetically arranged except for Figure 14.

PLATE II


13
11
12
T
A
O
TS
S
O
14
9
SC
V
VS
O
E
G
T
1
A
S
10
161.6 μ
200 μ
444.4 μ
T
O
M
T
P
Z
G
B
C
SC
S
180 μ
A
8

Aeverrillia Armata

(Figure 4)

Aeverrillia armata is transparent, yellowish, and horny, and occurs on *Laminaria Agardhii* and *Phyllophora membranifolia*. The latter alga was heavily encrusted with ten other bryozoan species and several algal species. *Aeverrillia armata* consists of numerous slender, paired autozoids arising from narrow stolons which cling closely to the plant but which can be pulled off as slender threads. This species is very similar to *A. setigera* which was discussed very fully in a previous study (Rogick, 1945a), except that it lacks the basal clasping processes of *A. setigera*. The polypides have eight tentacles in both species of the genus.

Aeverrillia Setigera

This delicate bryozoan was found growing inconspicuously on eight algal species. It clings closely to the plant thallus. It was pictured adequately in the previous study (Rogick, 1945a), so no figure of it is here included. The resemblance between it and *A. armata* is so close that one could easily mistake the one for the other.

Alcyonidium Polyoum

(Figures 5–7)

The various Alcyonidia are difficult to tell apart. The present *Alcyonidium polyoum* forms a firm gray or sometimes slightly yellowish crust around the hold-

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Plate II

Figure 8. *Bowerbankia gracilis*. An uncrowded stolonate colony of nine full-grown zoids (Z) and five smaller buds (B), growing on an algal filament (A). Other structures shown are: (C) caudal process; (G) gizzard; (M) parieto-vaginal musculature; (O) squared orifice; (P) polypide; (S) stolon; (SC) setigerous collar. Collection site and date same as for Figure 7.

Figure 9. *Bowerbankia imbricata*. Upper part of an extruded polypide showing ten tentacles (T) which upon retraction can be withdrawn into the tentacular sheath (TS). Around that is a stiff transparent setigerous collar (SC) which in turn can be withdrawn into the vestibular sheath (VS). Some debris has accumulated on the edge of the squared orifice (O). From Glen Island, New Rochelle, N. Y. on IX–16–1945.

Figure 10. *Bowerbankia imbricata*. A crowded colony which was scraped from *Chondrus crispus*. Collection date and site the same as for the preceding figure. Three of the long zoids have their tentacles extended. Three smaller ones have their setigerous collars partly extruded. Three zoids are shown with the polypides within them. The following parts are labelled: (E) esophagus; (G) gizzard; (I) intestine; (S) stolon; (T) tentacles.

Figure 11. *Bugula cucullifera*. Four zooecia, each provided with four spines (S). The upper three zoids show, at the side of the opesium (A), the remains of the short peduncle which had borne an avicularium. From Provincetown, Mass., on VIII–18–1947; Dr. M. Doty collector.

Figure 12. *Bugula cucullifera*. Three fertile zooecia topped with ovicells (O). The middle zooecium bears an avicularium (V), the other two have lost theirs. (A) is the opesium. The second row of zooecia which normally would be at the side of these zooecia was incomplete and was therefore not shown here. Same collection date and site and drawn to the same scale as the preceding figure.

Figure 13. *Bugula flabellata*. A freehand sketch, showing the close tuft-like mode of colony growth. About natural size.

Figure 14. *Bugula turrita*. A freehand sketch, showing the dainty, open spiral mode of colony growth. About natural size.

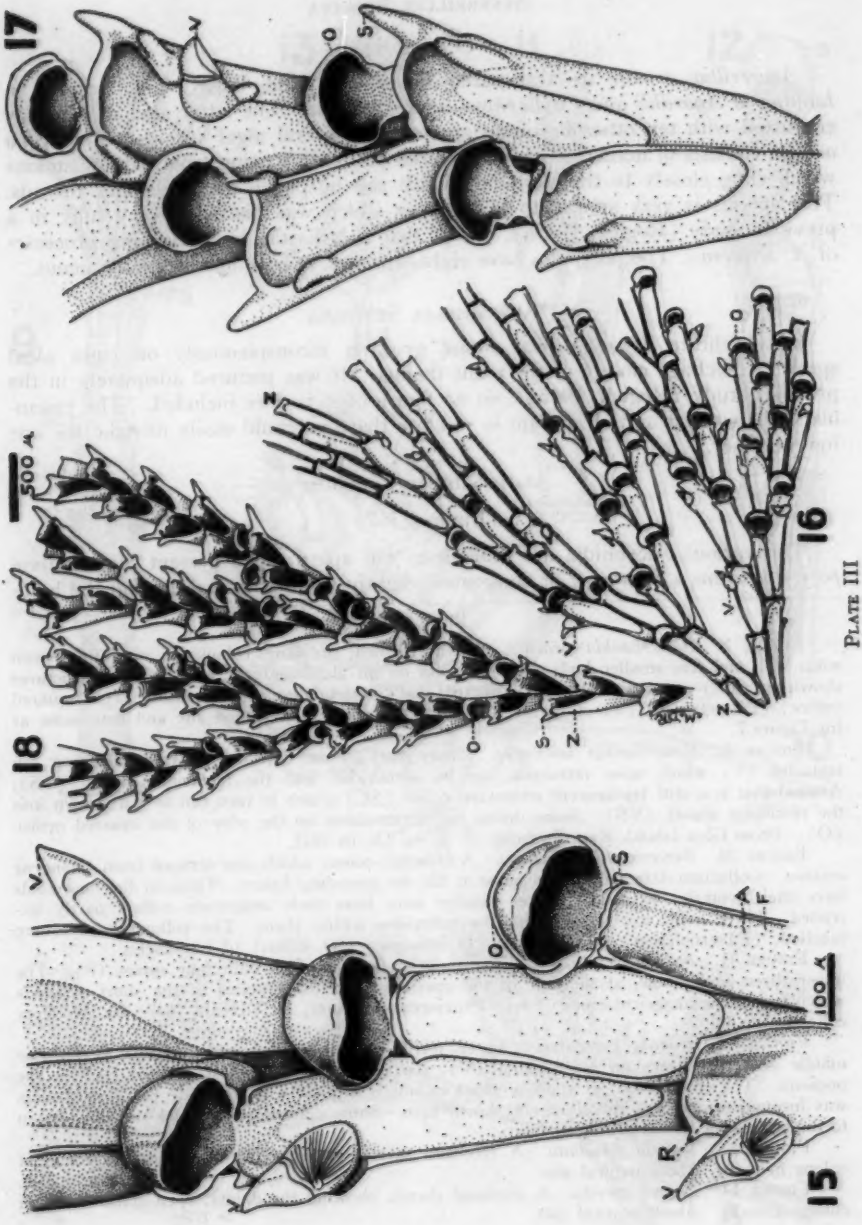

PLATE III

fasts, stalks, and blades of at least five algal species. Prenant and Teissier (1924, pp. 23, 27) reported Alcyonidium from Ascophyllum, Chondrus, Fucus, Himanthalia, *Laminaria* (*saccharina?*) and *Saccorhiza bulbosa*. Sometimes it coats the entire alga, using the various branches as cores around which to grow. The colony is rubbery to the touch.

The polypides had 16 tentacles. Measurements for 18 zoids ranged thus: zoid length 0.36–0.648 mm. and zoid width 0.24–0.504 mm. These are similar in range to figures given by Harmer (1915, pp. 37–38). The extremes in tentacle number given by various authors are 12 (Harmer, 1915, p. 38) to 20 (Silén, 1942, p. 11).

Bowerbankia Gracilis

(Figure 8)

Bowerbankia gracilis is very common. It forms a soft grayish furry mass on 18 algal species. It consists of a number of transparent tubes clustered along a stolon, sometimes so densely that the stolon is scarcely visible. Caudal processes appear on some zooecia. The eight tentacles can be counted only when the animal is alive and in the expanded state. The zoids in Figure 8 are in the retracted state with the tentacles and gut (collectively called "polypide") withdrawn into the body cavity. Under such conditions the squared orifices show nicely.

Bowerbankia Imbricata

(Figures 9–10)

Bowerbankia imbricata was found on only five algal species by the authors. Additional species on which it has been reported are: *Ascophyllum nodosum* (Adams, 1800, p. 11), *Corallina officinalis* (Hincks, 1880, p. 521), *Cystoseira fibrosa, Fucus serratus* (Joliet, 1877, p. 294), *Desmarestia aculeata,* and *Furcellaria fastigiata* (Thompson, 1840, p. 252). Colonies may cover extensive areas of several inches, coating the "stems" and thalli of Chondrus. They do not exclude other forms from growing on the alga but may grow among hydroids, sponges, and other encrusting forms.

Superficially, dense growths of *Bowerbankia imbricata* and *B. gracilis* are indistinguishable. Imbricata colonies whose zoids were filled with large ciliated

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Plate III

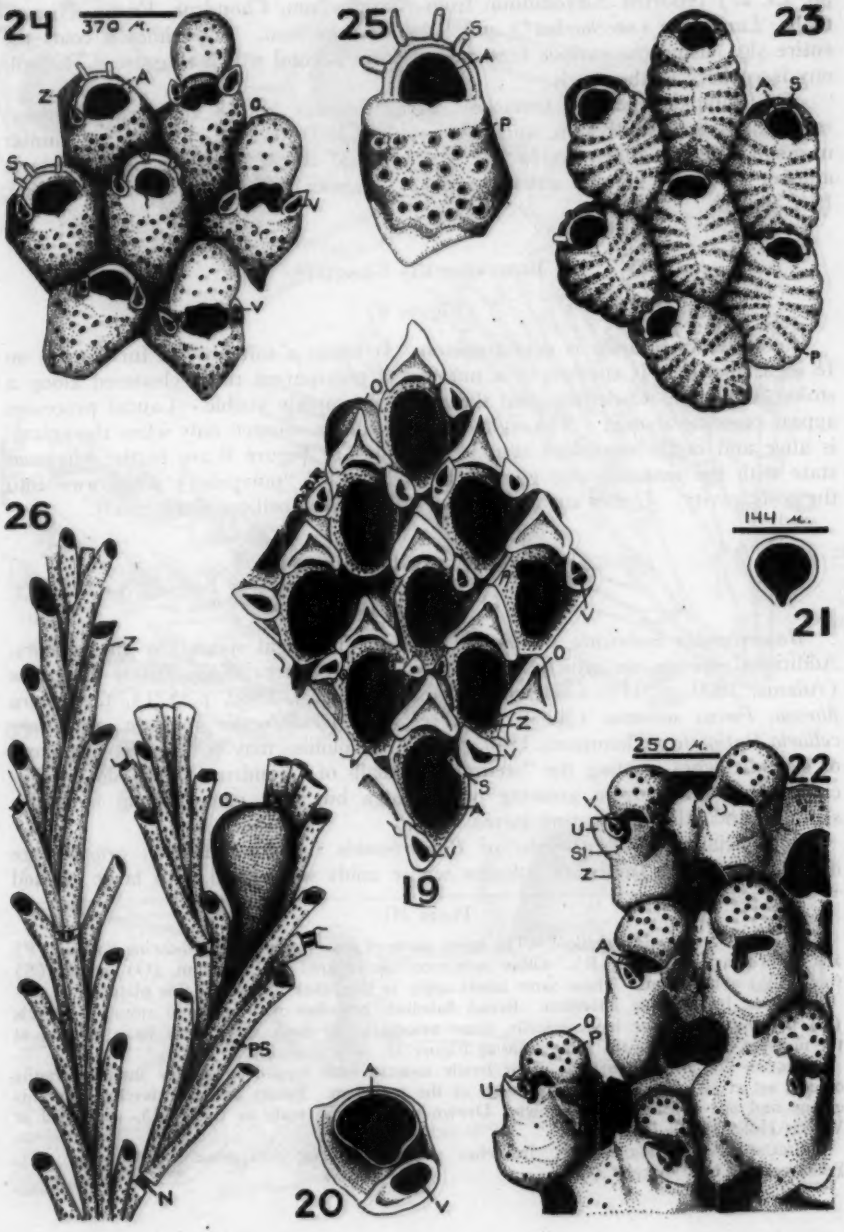
Figure 15. *Bugula flabellata.* The upper parts of three fertile ovicell-bearing zooecia (F) and an ordinary zooecium (R). Other structures shown are: (A) opesium, (O) ovicell, (S) spine, (V) avicularium. These same labels apply to the other figures on this plate.

Figure 16. *Bugula flabellata.* Broad flabellate branches with up to 6 rows of zooecia (Z) per branch. Some have ovicells, some avicularia, or both, and others have neither, at the moment. Drawn to the same scale as Figure 18.

Figure 17. *Bugula turrita.* Four fertile zooecia, each topped by a very shallow, fragile ovicell, set at an angle on the upper edge of the zooecium. Spines are well developed in this colony and one avicularium is shown. Drawn to the same scale as Figure 15. Collected at Woods Hole, VI–30–1938.

Figure 18. *Bugula turrita.* Branches showing biserial arrangement of zooecia (Z). Some zooecia bear ovicells (O).

PLATE IV


24
370 M.
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23
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A
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19
26
Z
O
PS
N
20
A
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144 M.
21
250 M.
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globular larvae were salmon pink in color because the red pigment of the larvae showed through the parent zoid walls. Such embryos were especially abundant in colonies collected during the first ten days in August (1947). Some embryos were found in colonies collected as late as August 31. Joliet (1877, p. 295) observed larvae during the month of July and reported that sexual reproduction took place from the end of June to early August.

Somewhat reniform larvae were released in great numbers on the morning of August 9, 1947. After a free-swimming period they attached to the substratum. Metamorphosis proceeded speedily, taking less than five minutes in some cases. The red color became concentrated at one end of the metamorphosing larva.

Adult zoids generally have ten tentacles and are square-topped when retracted. Measurements of seven retracted zoids were as follows: zoid length 0.925–1.374 mm.; zoid width 0.178–0.291 mm.; stolon diameter 0.040–0.101 mm.

Some very young colonies consisting of only one or two developing zoids had nine tentacles but their development could not be followed beyond a few days, so it could not be determined if these in time would increase their tentacular number to ten.

Bugula Cucullifera

(Figures 11–12)

Small fragments of this Bugula were found on *Fucus evanescens* and *Rhodymenia palmata* from Vineyard Sound on VIII–1–1945, on *Laminaria longicruris* from Provincetown, Mass., on VIII–18–1947 and on *Phyllophora membranifolia,* along with much *Crisia eburnea* and *Aetea sica* from New Bedford Harbor, on VIII–8–1945.

Plate IV

Figure 19. *Callopora aurita.* Nine zooecia (Z) each capped by a rounded ovicell (O) which is decorated by a raised triangular ridge. Spines (S) and avicularia (V) are present near the large opesia (A). The same letters apply to the other figures on this plate. A calcined specimen from which all the soft tissues have been burned away. Drawn to the same scale as Figure 24.

Figure 20. *Cellepora dichotoma.* Part of a very lightly calcified specimen showing the shape of the aperture and the position of the avicularium. From Nobska Beach driftweed, Woods Hole, VII–25–1944. Drawn to the same scale as Figure 21.

Figure 21. *Cellepora dichotoma.* Aperture of a very young zooecium.

Figure 22. *Cellepora dichotoma.* Portion of a moderately calcified colony. An avicularium is borne on the side of the umbo (U) and faces toward a sinus (SI) in the peristome or raised shelf encircling the front of the aperture. The ovicells have pores (P).

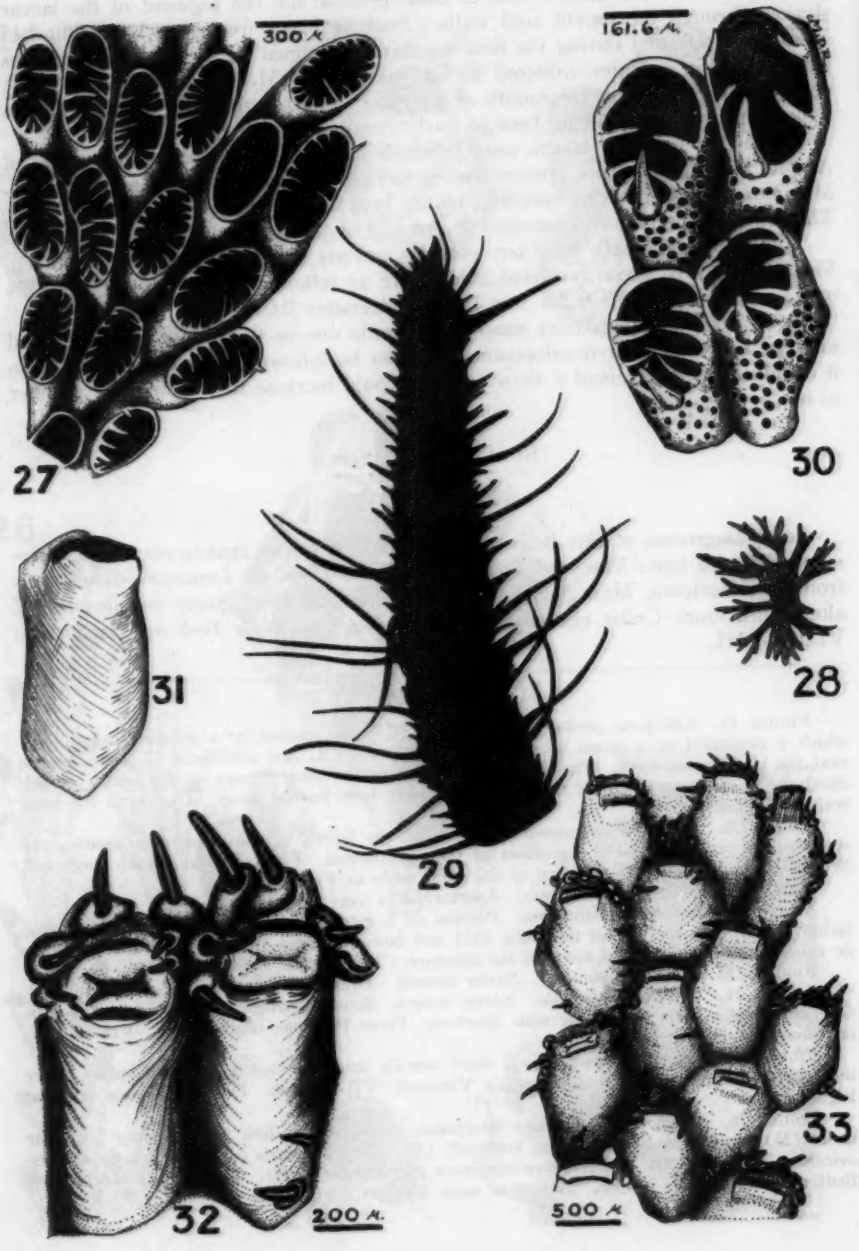
Figure 23. *Cribrilina annulata.* Seven zooecia. Drawn to the same scale as Figure 24.

Figure 24. *Cribrilina punctata.* Seven zooecia, three of which are capped by ovicells. One or two avicularia border the wide aperture. From Penikese Island, Mass., VIII–3–1947, on *Chondrus crispus.*

Figure 25. *Cribrilina punctata.* A more heavily calcified zooecium with 4 spines above the aperture. From Gay Head, Martha's Vineyard, VII–30–1946. Drawn to same scale as Figure 21.

Figure 26. *Crisia eburnea.* Four internodes, separated by dark yellow horny joints or nodes (N), bear a number of tubular autozoids (Z). One internode bears the greatly swollen ovicell or ooecium. The zooecia have numerous pseudopores (PS). From Black Rock, New Bedford Harbor, VIII–8–1945. Drawn to same scale as Figure 24.

PLATE V



Some embryo-filled ovicells were present. Very long rhizoid processes grew from the basal part of some of the colonies. Thirteen tentacles were counted on one zoid.

Bugula Flabellata

(Figures 13, 15, 16)

A small colony of *B. flabellata* was found on *Fucus vesiculosus*. It was far less common than *B. turrita*. Also, it seemed to prefer attachment to piles, live cars, and other submerged wooden objects rather than to algae. It is a very sturdy form, growing in thick, fan-shaped, yellow-orange tufts (Fig. 13) which are about a half inch tall.

Glass slides submerged in Eel Pond at Woods Hole from August 13 to August 31, 1945, were heavily overgrown with various animal forms, including *Bugula flabellata*. Colonies of the latter were by then about ¼ inch tall and contained hundreds of zoids.

According to Grave (1933, p. 384) its breeding season is between June 1 and November 15.

Bugula Turrita

(Figures 14, 17, 18)

Bugula turrita is very common, growing on at least 16 algal species. In general appearance it is more plant-like than animal-like. It is of yellow-orange color and has a soft, fluffy, but firm texture. It has a beautifully spiralling manner of growth (Fig. 14). The colony branches into a number of spiralling "turrets." Some of the colonies may be 1¼ inches tall.

The tentacle number is about 14.

Ovicells were seen in colonies collected from the end of June through mid-August (Fig. 17). Many young colonies developed from released larvae during that time.

Plate V

Figure 27. *Electra hastingsae*. Fifteen zooecia from the central part of a colony. One zooecium has lost all the spines around its opesium. The others have retained a varying number. Calcined specimen.

Figure 28. *Electra hastingsae*. A freehand sketch showing the flat, spray-like mode of growth which is so characteristic of this species. About natural size.

Figure 29. *Electra pilosa*, long-spined form. Tip of an alga, *Desmarestia aculeata*, completely encased by a bryozoan colony some of whose zooecia show an unusually long median spine. Shown in silhouette. Collected off Gay Head, Martha's Vineyard, VII–30–1946. Drawn to the same scale as Figure 33.

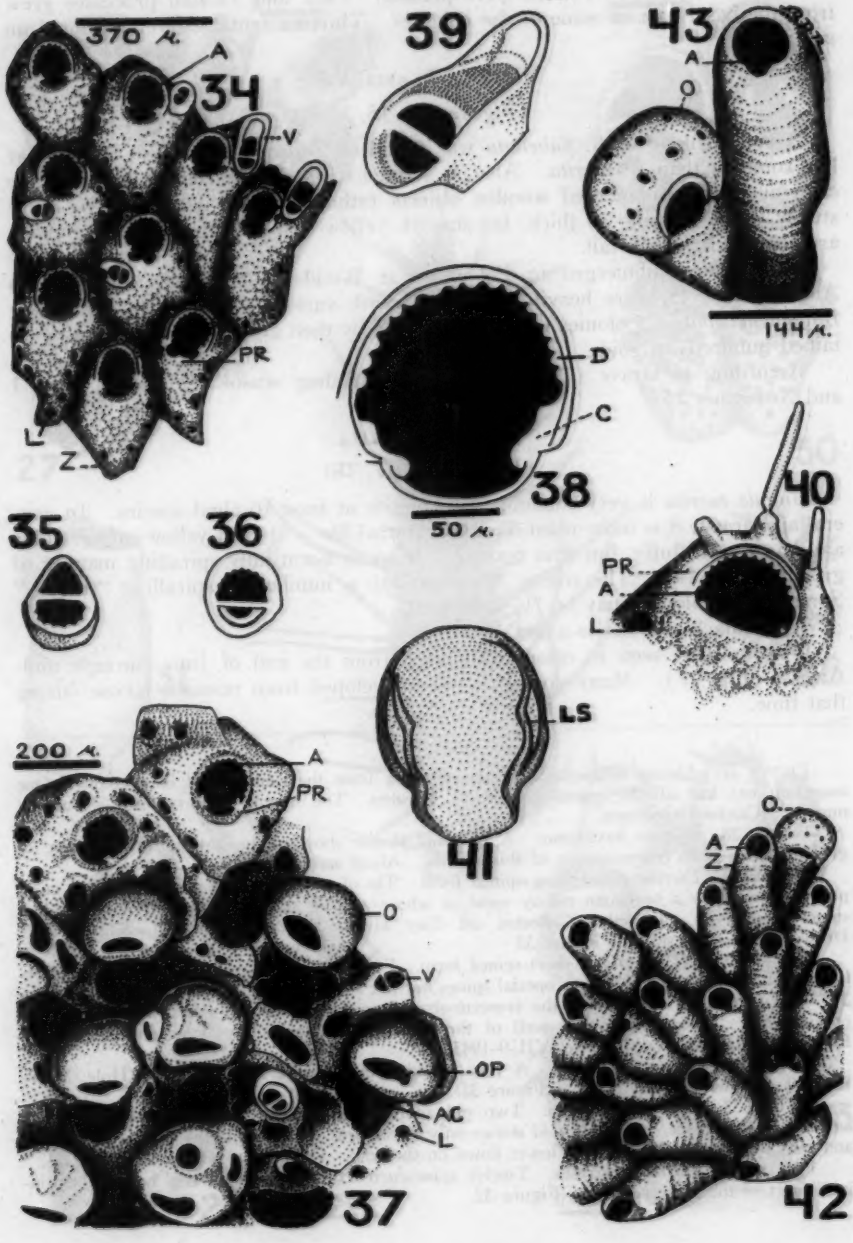
Figure 30. *Electra pilosa*, short-spined form. Four zooecia whose lowest, median spine is heavier and longer than the other opesial spines but not so long as the spines pictured in Figure 29. The two upper zoids show the crescent-shaped operculum rim in the upper part of the opesial area. The lower frontal wall of the zooecium is marked by numerous tremopores. From Devil's Foot, Woods Hole, VII–9–1945.

Figure 31. *Flustrella hispida*. A very young, spineless zoid. From Woods Hole, VIII–15–1939. Drawn to same scale as Figure 32.

Figure 32. *Flustrella hispida*. Two old zoids showing heavy "chitinization" of spines and lips of the orifice. The left zoid shows only circumoral spines while the right shows those and also additional spines located lower down on the zoid. From same colony as Figure 31.

Figure 33. *Flustrella hispida*. Twelve spine-encircled zoids from a less heavily "chitinized" part of the same colony as Figure 32.

PLATE VI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O
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50 μ.
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36
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PR
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41
200 μ.
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L
37
O
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Z
42

Callopora Aurita

(Figure 19)

Callopora aurita was not abundant on algal material, being found more commonly and in more extensive patches on rocks. Very small white colonies were found on specimens of *Phycodrys rubens* collected from Rye Ledge, Rye, New Hampshire, on IX–30–1945; on *Phyllophora membranifolia* dredged from Great Harbor, Woods Hole, Mass., on VIII–8–1946, and on holdfasts of *Laminaria Agardhii*. The colonies form a fine encrusting calcareous mesh on the algal thallus.

Ovicells were present in the colonies, but it was not possible to determine whether they were tenanted by larvae at the time of collection. Twelve tentacles were counted on one zoid.

Cellepora Dichotoma

(Figures 20–22)

Its small, white, calcareous zoaria grow on *Chondrus crispus, Gracilaria confervoides, Phyllophora Brodiaei* and *P. membranifolia*. Its zoids are crowded

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Plate VI

Figure 34. *Hippoporina contracta*. Portion of a young, uncrowded colony showing nine zooecia (Z), four of which are without avicularia (V) and three of which have a small rounded avicularium and two of which have spatulate avicularia (V). The distinctive serrate aperture (A) is readily distinguishable in these not heavily calcified zoids. The peristome (PR) is prolonged into a small bump or mucro below the aperture in the central zoid. Areolae (L) border each zooecium. The same labels apply to the other figures on this plate. Specimens dredged from Great Harbor, Woods Hole, on VIII–8–1946.

Figure 35. *Hippoporina contracta*. A small, slightly pointed avicularium with part of its aperture serrated. Drawn to same scale as Figure 43.

Figure 36. *Hippoporina contracta*. A small rounded avicularium with part of its aperture serrated. Drawn to same scale as Figure 43.

Figure 37. *Hippoporina contracta*. A more crowded and calcified colony than that of Figure 34. The upper two zoids show the typical serrate aperture. Seven ovicells (O) with large, comma-shaped pores (OP) are visible. Beneath each ovicell is a large peristomice (AC) or peristomeal opening (here shown in black), at the bottom of which lies the distinctive serrate aperture (invisible in this picture). Calcined specimen.

Figure 38. *Hippoporina contracta*. The serrate aperture characteristic of this species. The aperture, black in this calcined specimen, in life is closed over by an operculum which is pictured in Figure 41. The aperture has 14 to 18 small rounded denticles (D) and two large bifid cardelles (C).

Figure 39. *Hippoporina contracta*. A spatulate avicularium seen at an angle. Drawn to the same scale as Figure 43.

Figure 40. *Hippoporina contracta*. Upper half of a zooecium showing areola (L), denticles, cardelles, peristome (PR) and three spines above the aperture (A). Drawn to same scale as Figure 43.

Figure 41. *Hippoporina contracta*. Operculum which closes the aperture of the zooecium. It has a stiffened rim and lateral sclerites (LS). Drawn to same scale as Figure 38.

Figure 42. *Hippothoa hyalina*. A colony showing a number of ordinary zooecia (Z) and a dwarfed one topped by an ovicell (O). Drawn to same scale as Figure 34.

Figure 43. *Hippothoa hyalina*. Another view of the punctate ovicell, its dwarfed zooecium and a normal sized zooecium. The latter shows the typical aperture, rounded and with a sinus. The transverse grooving normally found in the zooecia is faintly indicated in the larger zoid. Specimens dredged off Gay Head, Martha's Vineyard, VII–30–1946.

against each other. Embryo-filled ovicells were present at the time of collection (July 25, 1944).

There is some question as to the classification of this species. *Cellepora americana, Cellepora avicularis* and *Cellepora dichotoma* show such integradation that their exact status or validity needs critical review by some future worker. The species of the present study is identical with Marcus' illustration of *C. dichotoma* (Marcus, 1938, Plate XI, Fig. 26).

The species characteristics are as follows: (1) peristome with a sinus next to a raised umbo on the side of which is an avicularium facing the sinus; (2) aperture rounded, with postral sinus; (3) ovicell with pores, rounded and somewhat flattened; and (4) a few small pores (areolae) around the frontal wall of the zooecium.

A heavily calcified zoarium may show the ovicells almost completely immersed on all sides except the frontal in the secondarily calcified zooecial wall. The frontal of such ovicells is provided with good-sized pores and is at a lower level than the secondarily calcified outer zooecial wall.

Cribrilina Annulata

(Figure 23)

This encrusting species was very uncommon. Only one white calcareous zoarium was found on *Phycodrys rubens,* from Rye, N. H., on the reverse side of the thallus from the finer, more fragile *Cribrilina punctata.* Three or four spines were present around the aperture. Marcus (1940, p. 203) reported *C. annulata* from Laminaria.

Cribrilina Punctata

(Figures 24–25)

Small patches of this fragile white calcareous form were found encrusting seven algal species. The number of spines around the aperture varied from none to five. The frontal pores were somewhat irregular in size and position. This was not a very common form; only a few colonies appeared in the collection.

Crisia Eburnea

(Figure 26)

Crisia eburnea was exceedingly common on 18 algal species. It was especially abundant on *Chondrus crispus,* the two Phyllophorae, and *Phycodrys rubens.* A very large amount of it was collected from the driftweed along the beaches at Nobska, Gay Head, and Cuttyhunk. Dried specimens were just as useful as wet ones for taxonomic purposes.

Prenant and Teissier (1924, p. 18) reported *C. eburnea* from Halidrys and certain Cystoseiras.

It forms brittle, white, openly dendritic tufts up to 7 mm. tall on the thalli of the small, and around the holdfasts of the large, algae. A Crisia colony consists of a number of calcareous tubular zooecia forming internodes which are separated

from other internodes by short, narrow, yellowish to brown chitinous joints. A branch which consists of ordinary tubular zooecia (autozoids) alone is called a sterile internode. Three such are pictured in Figure 26. A branch which consists of a number of autozoids and a long, very swollen brood chamber (ooecium or ovicell) is called a fertile internode. One is pictured in Figure 26. In the identification of different species of Crisiidae the number of zooecia in the fertile and sterile internodes is important. In *Crisia eburnea* the sterile internode has four to eleven zooecia, and a fertile internode seven to ten (Borg, 1944, p. 158).

Ovicells were found on specimens collected throughout the summer months. Embryos were seen in some on August 8, 1946.

Cryptosula Pallasiana

Cryptosula pallasiana forms a round, flat, regularly patterned, pale orange to white encrustation on rocks, shells, and algae. It occurs more commonly and forms larger colonies on the harder substrates than on the algae but is not uncommon on the latter. It was found on eleven algal species which came from a number of collecting sites between Martha's Vineyard, Woods Hole, North Falmouth, and New Bedford (all in Massachusetts). They grew on the thalli of algae and on the Laminaria holdfasts. Colonies attached to *Enteromorpha intestinalis* and *Ulva Lactuca* var. *rigida* were young and small, consisting of few (five or less) freshly formed zoids (as of VIII–13–1945). Submerged glass slides, left in Eel Pond at Woods Hole for the first two weeks in July and kept a week longer in running sea water in the laboratory, were well covered with many animal forms including *Bugula turrita, Pedicellina cernua* and *Cryptosula pallasiana*. The Cryptosula colonies had from one to thirty zoids on these slides. Their polypides had 16 tentacles.

Barrois (1877, p. 139) reported larvae in August and September. The Woods Hole specimens produced larvae in those months as well as during June and July.

Joliet (1877, p. 291) reported this bryozoan on *Callothrix pannorum*. Prenant and Teissier (1924, p. 23) reported Cryptosula from other Laminariae, Himanthalia, and *Saccorhiza bulbosa*.

No drawings of Cryptosula are here included because the species was previously figured (Rogick, 1945b, p. 3, Fig. 1).

Electra Hastingsae

(Figures 27–28)

A few small colonies of *E. hastingsae* encrusted the thalli of *Fucus vesiculosus* var. *spiralis* and *Laminaria Agardhii*. Marcus (1938, p. 17) reported the bryozoan from Zostera. Sometimes it grows on the gill chamber of Libinia crabs. Generally, however, the bryozoan is found on hard substrates (rocks and shells) more often than on algae.

Electra hastingsae is a fragile, white, calcareous species, forming completely adherent frond-like traceries on the substratum (Fig. 28). Some colonies lack spines around the opesium. Other colonies have a variable number of very delicate ones, sometimes as many as 18. Some of the spines may break off (Fig. 27).

A new zooecium may occasionally grow right out of the opesium of another empty one. Whether that is a case of regeneration or the settling of a new larva on an old colony, is not certain. Embryos were not observed.

Electra Pilosa

(Figures 29–30)

Electra pilosa is an extremely common calcareous but fragile encrustation on 17 algal species. It has been reported previously from: *Fucus serratus* (Joliet, 1877, p. 290); Ulva (Hutchins, 1945, p. 540); *Laminaria saccharina* (Leidy, 1855, p. 9); Furcellaria and Polyides (Marcus, 1940, p. 118); the Cystoseiras, Corallina (Prenant, 1927, p. 24) and Zostera (Prenant, 1932, p. 92).

Electra pilosa forms grayish-white, single-layered colonies which spread like a fine, closely-woven mesh over large areas, sometimes a foot in length, of algal thalli. Laminaria and Rhodymenia thalli are particularly favored. Numerous colonies may grow toward and into each other to form an almost continuous thin crust over the thalli. The lacy *Plumaria sericea* fronds, in some instances, were completely encased in *Electra pilosa*. Many Foraminifera were scattered over the Electra.

Great variation in degree of spination occurs. Several *E. pilosa* "forms" of dubious validity are mentioned in literature: forma *typica*, f. *dentata*, f. *laxa* and f. *verticillata*, differing slightly from each other, mainly in the presence or length of the principal median proximal spine. Borg (1930, p. 63) and others mentioned that occasionally several of these growth forms may be found in a single *E. pilosa* colony, and therefore should not be considered valid varieties.

The present writers found both long-spined (forma *verticillata*, Fig. 29) and short-spined (forma *dentata*, Fig. 30) growths in the collections, the latter being far more common than the long-spined specimens.

Tentacles numbered 12 to 14.

Flustrella Hispida

(Figures 31–33)

Flustrella hispida grows on five Woods Hole algal species: *Ascophyllum nodosum, Chondrus crispus, Fucus vesiculosus, Phyllophora membranifolia* and *Ulva Lactuca* var. *rigida*. Also, the M. B. L. Collecting Crew has on numerous occasions brought in Ascophyllum covered with Flustrella from other localities. Additional algae from which it has been recorded are: *Gigantina mamillosa* (Hincks, 1880, vol. 1, p. 507); *Fucus serratus* and Cystoseira (Joliet, 1877, p. 292). It was far more common on Ascophyllum and Fucus than on the green or red algae in the Woods Hole region.

Flustrella hispida forms a brown, rubbery, and somewhat slimy crust over extensive areas of the algal thallus. The zoids are fairly soft and baggy (Figs. 31, 32). Thirteen tentacles were counted on one specimen. Spines were lacking in the very youngest zoids (Fig. 31), but more mature ones show variation in distribution and number of spines (Figs. 32, 33). In the oldest parts of the colony the spines may become very thick and dark reddish brown, and appear mounted on horny pads (Fig. 32) about the zooecial orifices. In younger colonies, and also in some older zooecia, as in the right zoid of Figure 32, spines appear elsewhere about the zoid than just around the orifice. The reinforced orifices are shaped like the top of a purse (Figs. 32, 33).

Barrois (1877, p. 214) found *F. hispida* colonies filled with embryos during the months of May, June and July.

Hippoporina Contracta

(Figures 34–41)

White to buff-colored colonies of this species were found more often on rocks and shells than on algae. However, some did grow on *Phyllophora Brodiaei* and *P. membranifolia,* and were up to 2 cm. in diameter. The appearance of the colony varies greatly, depending upon the age of the colony, degree of calcification, the presence of ovicells and the nature of the substratum (compare Figs. 34 and 37).

The key character in identifying this species is the "beaded" aperture (Fig. 38) whose circular outline, serrate antral border, and two bifid cardelles marking the postral border vary so little that they can be identified in either old or young colonies. The number of rounded denticles in the antral border ranges from 14 to 18. In old, heavily calcified colonies (Fig. 37), the zooecial wall and peristome around the aperture may increase in thickness so greatly that the primary "beaded" aperture comes to lie considerably below the external body wall surface, at the bottom of a calcareous "well," the wall of which is formed by the peristome. The top opening of this calcareous "well" is called either the secondary aperture or the peristomice (Fig. 37, AC).

In younger, less calcified colonies, two to six oral spines, sometimes measuring 0.12–0.13 mm., may appear on the peristome (Fig. 40). These break off and their bases may become completely overgrown in the process of increasing calcification of the body wall.

Marcus reported 12 tentacles for this species (1937, p. 98).

The ovicells are quite characteristic also. They are smooth, hemispherical, and provided with a large, comma-shaped membranous area or pore (Fig. 37, OP) on the frontal surface.

Six to thirteen marginal pores or areolae (Fig. 34, L) can be seen in the zooecial body wall.

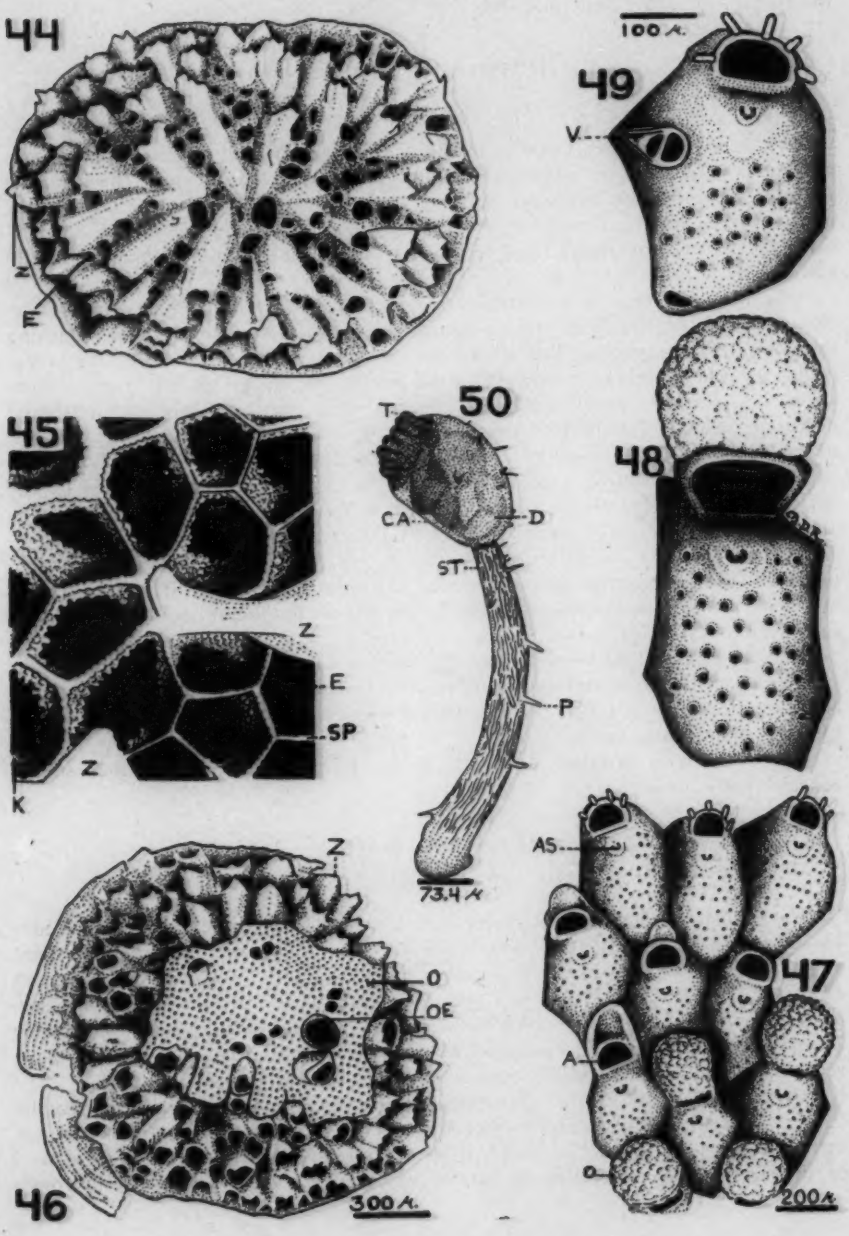
Hippothoa Hyalina

(Figures 42–43)

Hippothoa hyalina was extremely common on 17 algal species in the Woods Hole region. Borg (1930, p. 84) listed it from *Laminaria saccharina;* Prenant and Teissier (1924, p. 22) from the Florideae and Dictyota, and Prenant (1927, pp. 26–27) from *Laminaria cloustoni* and *Saccorhiza bulbosa.* A dry herbarium mount of a pressed *Phycodrys rubens* (collected by Dudley at Marble Head in Sept. 1874) was examined by the writers and found to contain easily recognizable and uncrushed *H. hyalina* zoaria.

Hippothoa hyalina forms glistening white or grey calcareous patches usually from 1 to 8 mm. in diameter, either on or encircling the thalli of most of the mentioned algae and on the holdfasts of Laminaria and Rhodymenia. "Stems" or filaments of *Cystoclonium purpureum* var. *cirrhosum* were encased in rough

PLATE VII


44
N
E
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49
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73.4 μ
48
Z
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46
300 μ
AS
47
A
O
200 μ

calcareous sheaths of *H. hyalina* sometimes an inch in length. Often the sheaths of colonies were arranged in a linear series, the total series attaining a length of several inches.

Embryo-filled ovicells were plentiful in specimens collected during July and August in the Woods Hole area. Many ancestrulae or the single individuals from which a colony begins were observed in collections made up to August 11, 1945. These ancestrulae arise from sexually produced larvae. Barrois (1877, p. 164) remarked that at Roskoff the embryos were carried in transparent ovicells in the months of May and June, so apparently the breeding season is of considerable length.

Lichenopora Hispida

(Figures 44–46)

One fertile and less than a dozen small immature colonies of this species were found growing on *Phycodrys rubens* and *Phyllophora membranifolia* collected from Rye, N.H. on IX–30–1945.

The fertile colony (Fig. 46) has a brood chamber provided with a thin, rounded aperture and many small pores. The autozoids (Fig. 44) terminate in jagged edges. They are partly surrounded by reticulate alveoli (Fig. 45) which are lined with small calcareous projections from the interalveolar septa whose thickness is variable.

Borg (1926) gives a good account of the development of various Cyclostomata, including the Lichenoporae, and discusses the terminology of the group.

Membranipora Lacroixii (?)

Membranipora lacroixii is a species whose identification and synonymy are exasperatingly confused in literature. Part of this is due to vague original

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Plate VII

Figure 44. *Lichenopora hispida.* A fairly young colony showing autozooecia (Z) separated by large cavities or alveoli (E). Drawn to same scale as Figure 46.

Figure 45. *Lichenopora hispida.* Detail of the center of an immature though fair-sized colony, showing about 17 alveolar spaces (E), the interalveolar septa (SP) between them and the projections (K) from the calcareous cryptocyst of the septal wall. The sides of two autozoids (Z). Drawn to same scale as Figure 49.

Figure 46. *Lichenopora hispida.* A damaged, fertile colony, showing an irregular, punctate brood chamber (O), the brood chamber aperture (OE) and numerous short (immature or damaged?) and some normal autozoids (Z).

Figure 47. *Microporella ciliata.* Nine zooecia, four of which have well-developed ovicells (O) and three of which have shelf-like beginnings of ovicells distal to the aperture (A). Each zooecium has a crescent-shaped ascopore (AS) and smaller frontal pores. Oral spines occur on the upper three zoids. The crescent-shaped ascopore and the hemispherical aperture are key characters for this species.

Figure 48. *Microporella ciliata.* A zooecium topped by an ovicell. Both have pores but of different size. Drawn to same scale as Figure 49.

Figure 49. *Microporella ciliata.* A zooecium showing oral spines and a pointed avicularium (V) in a characteristic position. The avicularium may develop on either the right or left side of the zoid. Same collection area and date as Figure 43.

Figure 50. *Pedicellina cernua.* A single zoid consisting of a stalk (ST) and a calyx (CA) containing the polypide (D) and rolled in tentacles (T). A few spines (P) occur on the stalk and calyx. From Black Rock, New Bedford Harbor, VIII–8–1945.

PLATE VIII

descriptions, and part due to the apparently great variation in spination and degree of calcification of the zooecia. Such will continue to be the state of affairs until someone takes the trouble to make a very elaborate study of the variations of this species. The Woods Hole and New Rochelle specimens of the present study resemble the *Membranipora lacroixii* pictured by Osburn (1912, Plate 22, Fig. 22), the *Conopeum lacroixii* pictured by Canu and Bassler (1920, Plate 13, Fig. 9), the *Conopeum reticulum* pictured by Harmer (1926, Plate 13, Fig. 12), the *Biflustra aciculata* of MacGillivray (1891, Plate 9, Fig. 5) and the *Membranipora crustulenta* of Osburn (1944, Fig. 20, p. 32). Our specimens differ from the *Conopeum reticulum* pictured by Marcus (1938, Plate 2, Fig. 5A), the *Conopeum lacroixii* figured by Canu and Bassler (1923, Plate 29, Fig. 4), the *Membranipora reticulum* f. *lacroixii* and *M. crustulenta* of Borg (1930, pp. 63–65). The present study specimens definitely are not the *M. crustulenta* of Borg because that species is pictured with a calcified operculum, a character not present in our specimens. Until the status and limits of the species are fixed, the present authors will continue to call it *M. lacroixii*, as in Osburn's 1912 paper.

Membranipora lacroixii was found encrusting rocks, shells, and less frequently the algae *Ascophyllum Mackaii, Chondrus crispus* and *Phyllophora membranifolia*. It formed a delicate, gray-white tracery which adhered so closely to the substratum, especially rocks, that it was difficult to dislodge. No avicularia or ovicells were found. The conspicuous triangular spaces mentioned as characteristic by Harmer and Marcus were not observed on our specimens. Calcifica-

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PLATE VIII

FIGURE 51. *Schizoporella biaperta*. Nine regularly arranged, moderately calcified zooecia, one of which is without an avicularium. Drawn to the same scale as Figure 54. Calcined specimen.

FIGURE 52. *Schizoporella biaperta*. A small ellipsoidal avicularium. Drawn to same scale as Figure 55.

FIGURE 53. *Schizoporella biaperta*. A small oval or somewhat pointed avicularium. Drawn to same scale as Figure 55.

FIGURE 54. *Schizoporella biaperta*. A fertile area of a colony showing six ovicells. The middle ovicell is most nearly typical in appearance. The frontal area of its zooecium is more highly calcified than that of the other three zooecia above it, and than that of the zooecia of Figure 51.

FIGURE 55. *Schizoporella biaperta*. A moderately calcified zooecium showing two avicularia, the two apertural teeth (cardelles) and the sinus between them. A typical specimen.

FIGURE 56. *Schizoporella biaperta*. A heavily calcified zooecium topped by an ovicell. The zooecial shape is atypical and due to crowding in the colony and to excessive calcification. The avicularium is heavily calcified. The depressed rim of the ovicell frontal has been accidentally over-emphasized and should look less depressed (see Figure 54, middle ovicell). Drawn to same scale as Figure 55.

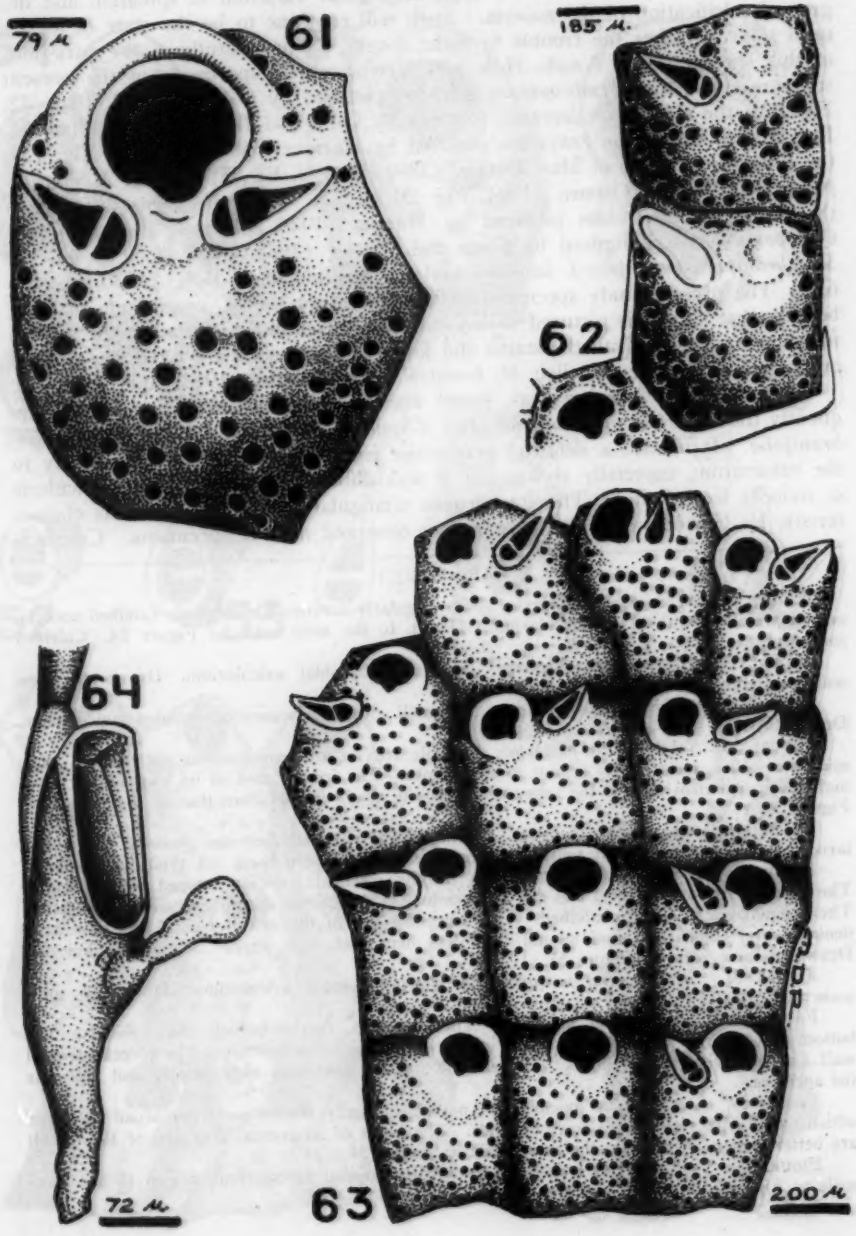
FIGURE 57. *Schizoporella unicornis*. A sharply pointed avicularium. Drawn to same scale as Figure 55.

FIGURE 58. *Schizoporella unicornis*. Three zooecia, two of which have ovicells. The bottom zooecium is twice as broad as the other two to which it gives rise. The zooecial frontal wall and the ovicells have pores. The sharply pointed avicularia vary in size and are near the aperture.

FIGURE 59. *Schizoporella unicornis*. Another unusually shaped and very broad zooecium which would give rise to two rows of zoids. The pores of its frontal area and of the ovicell are better shown. Drawn to the same scale as Figure 54.

FIGURE 60. *Schizoporella unicornis*. A smaller pointed avicularium, drawn to the same scale as Figures 55 and 57.

PLATE IX



tion was not heavy. Some New Rochelle specimens had up to ten spines, while others had no spines around the aperture—all in the same colony. Some zoids had 11 tentacles.

This species was pictured in an earlier paper (Rogick, 1940, p. 167, Figs. 6-9).

Membranipora Tuberculata

A specimen of *Membranipora tuberculata* was found on fronds of Cryptopleura sp. and Sargassum sp. which were sent to the writers by Dr. William Randolph Taylor. The Cryptopleura had come from Puerto de la Paloma, Uruguay, from the collection of Carmen de Franco de Pimienta. The Sargassum sp. had been collected by Adrien Questel on April 21, 1944, from Guadeloupe, Antilles. This Membraniporan has been previously reported by Marcus from Laminaria (1939, p. 126) and Fucus (1937, p. 34); by Hastings (1929, p. 706) from Padina; and by Osburn (1912, p. 231) from *Sargassum bacciferum* which had drifted into Vineyard Sound.

The extensive colonies of *M. tuberculata* spread flatly over the algal fronds in an ivory-white lacework, reminiscent of *Electra pilosa*. The two or three prominent calcareous tubercles at the anterior end of each zooecium from which this species gets its name may project separately and distally or may coalesce, forming a somewhat rounded ledge.

Since *M. tuberculata* was adequately pictured in both Osburn's (1912, as *M. tehuelca*) and Marcus' (1937) papers, no figure of it was included in the present study.

Microporella Ciliata

(Figures 47–49)

Small, flat, circular colonies, white to irridescent in color, calcareous though fragile, encrust shells, rocks, and five algal species in the Woods Hole region. Prenant and Teissier (1924, p. 23) found *Microporella ciliata* on three additional algae: Himanthalia, *Laminaria saccharina* and *Saccorhiza bulbosa*. Hadley Harbor specimens were found growing on the same thallus with Foraminifera, *Aetea sica, Crisia eburnea, Hippothoa hyalina,* and *Schizoporella biaperta*.

Plate IX

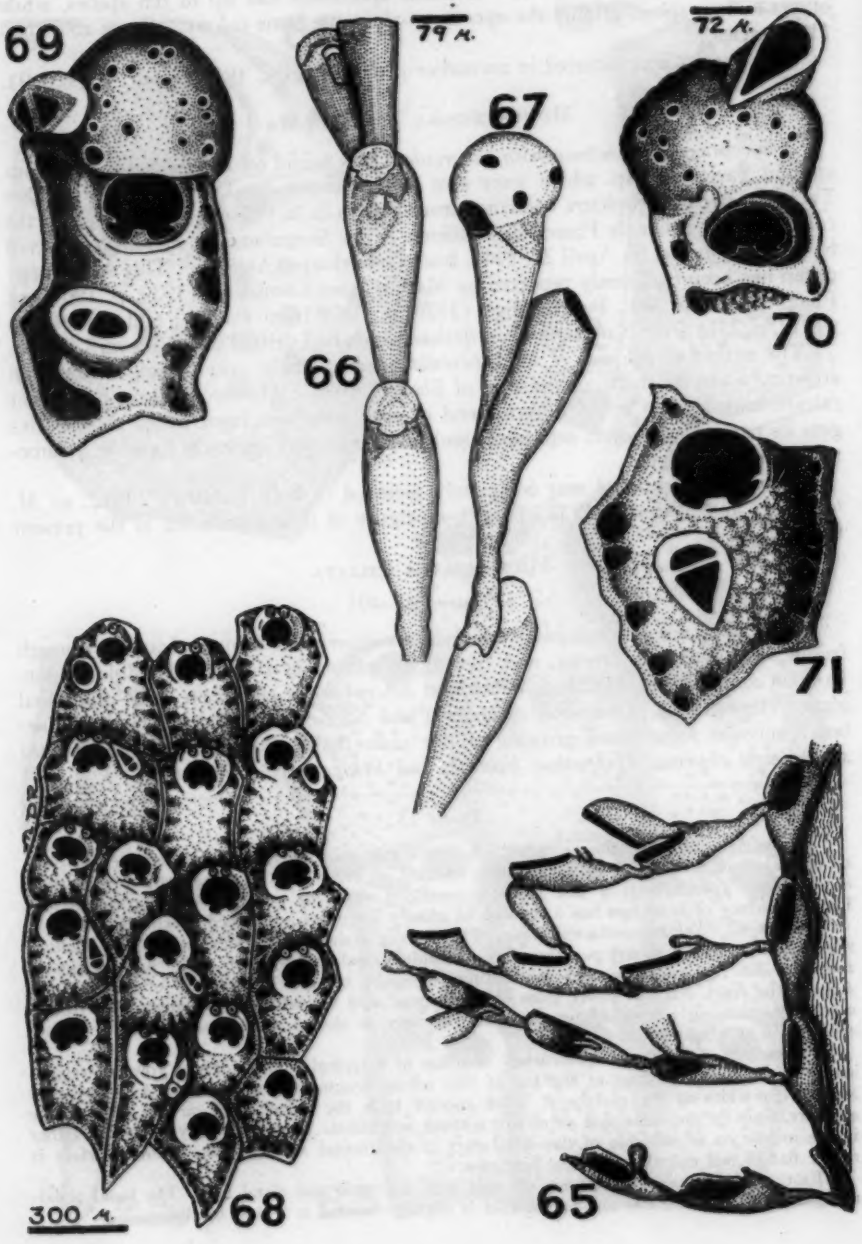
Figure 61. *Schizoporella unicornis*. A single zoid showing typical rounded aperture with its postral sinus and two pointed avicularia situated on either side of the aperture. The presence of two avicularia is a less frequent condition than the presence of one avicularium. Frontal surface of zooecium has a number of closely set pores. Calcined specimen.

Figure 62. *Schizoporella unicornis*. Three zooecia showing varying degrees of calcification. The uppermost square zooecium has a completely calcified aperture. The second squared zooecium has the aperture and avicularium openings completely calcified or plugged up. Around the right, left and lower sides of this second zoid are white septa, outgrowths from a newly overgrowing colony whose marginal zooecium is shown as the partial, third, bottom zooecium with black aperture.

Figure 63. *Schizoporella unicornis*. Portion of a typical colony. Three rows of zooecia are at the bottom and four at the top of this colony fragment, showing how a colony may increase in width at the periphery. Five zooecia have the avicularium on one side of the aperture, four on the other and three are without avicularia. Apertures may be placed either in the middle or at one side of the distal part of the frontal surface. The frontal surface is rather flat in this colony. Calcined specimen.

Figure 64. *Scruparia ambigua*. A zoid with a frontal and distal bud. The basal proximal part of the frontal bud and of the zoid is slightly twisted, a typical condition.

PLATE X



Some zooecia are without oral spines, avicularia or ovicells. Others have them. Oral spines may number from 3 to 7 (Figs. 47, 49). Ovicells are globose, "pebbled" in texture, and provided with small pores (Fig. 48). One avicularium is placed at an angle on either the right or the left frontal side, one-third to one-half of the way down and laterad from the aperture. The aperture is hemispherical and placed above the small crescent-shaped ascopore (Fig. 49). Canu and Bassler (1930, p. 47) reported 13 to 14 tentacles for this species.

Embryo-filled ovicells were collected on VIII–28–1939. Many very young colonies were found developing at that time also.

Pedicellina Cernua

(Figure 50)

Pedicellina cernua though small, soft-bodied, and inconspicuous was reported on 11 algal species. Also, Leidy (1855, p. 11) reported it on the "roots" of *Laminaria saccharina;* Prenant and Teissier (1924, p. 19) reported it on the Cystoseiras, Florideae and Dictyota; and Joliet (1877, p. 296) on *Corallina squammata* and *Cladophora rupestris.*

It has a creeping stolon from which arise flexible stalked zoids. Spines occurred on the calyx and stalk (Fig. 50) of a few zoids but most specimens were without them. The tentacle number in several very young zoids was 8 to 12. The number increases with age. Marcus (1939, p. 212) gives the tentacle range for this species as 8 to 24.

Plate X

Figure 65. *Scruparia ambigua.* A row of four zooecia encrusting a thick algal filament. From these arise four branches of zoids. The opesial rim of all but possibly the terminal budless individuals is parallel to the back of its own zoid. Drawn to the same scale as Figure 68.

Figure 66. *Scruparia clavata.* Two full-grown zooecia and two developing buds. The smaller, left bud belongs to a developing ovicell. The ovicelled individual originates below the other bud. Drawn to the scale above.

Figure 67. *Scruparia clavata.* Two grown zooecia and a third smaller one topped by a globose ovicell which has several large pores. The ovicell faces in the opposite direction from the ordinary zooecium. Drawn to same scale as Figure 66.

Figure 68. *Smittina trispinosa.* Eighteen ordinary zooecia growing in a very regular fashion. Some zooecia have rounded and some pointed avicularia while others have none at all. The flared peristome about the aperture is well developed in many. Some have spines, others do not. Calcined specimen.

Figure 69. *Smittina trispinosa.* A zooecium, ovicell and two avicularia. The circular aperture with the two cardelles (lateral teeth) and the lyrula (broader, median tooth) is a key character. So are the ovicell with its pores and leaning avicularium and the row of marginal zooecial areolae. Avicularia may be found in various locations on the frontal zooecial surface (compare this figure with Figures 68 and 71). The zooecium shows secondary calcification about the lower avicularium and partly covering the lower areolae. Drawn to same scale as Figure 70. Calcined specimen.

Figure 70. *Smittina trispinosa.* An ovicell topped by a triangular avicularium. The zooecial peristome is heavily calcified and forms a collar at the bottom of which can be seen the aperture, lyrula and cardelle.

Figure 71. *Smittina trispinosa.* A young zoid showing the characteristic apertural features and a frontal triangular or pointed avicularium. The frontal surface is slightly "beaded" and marginal areolae outline the thin edge. Drawn to same scale as Figure 70.

Schizoporella Biaperta

(Figures 51–56)

This white to reddish-orange bryozoan is fairly common on nine algal species. Its colonies attain a diameter of 2 cm. or more and appear fairly sturdy. They grow either flat on the thallus or may extend beyond the thallus, forming calcareous "ruffles" which may be lamellate (several layers in thickness).

The key characters of this species are: (1) rounded aperture with a postral sinus between the two cardelles (Fig. 55); (2) one or two small oval or ellipsoidal avicularia (Figs. 52, 53) mounted on mammillate prominences at the right or left or both sides of the sinus area (Figs. 51, 55); (3) frontal wall perforated by irregularly sized and spaced pores, and (4) hemispherical ovicells the edge of whose frontal area is slightly depressed and marked by faint calcareous ribs (Figs. 54, 56). Heavy calcification obscures some of these characters, especially the porous frontal area of the zooecium (Figs. 54, 56).

Twelve tentacles were counted on one zoid.

In July and August, the ovicells contained red embryos or larvae.

Schizoporella Unicornis

(Figures 57–63)

Schizoporella unicornis is very common on rocks and shells, but less frequent on algae. It grew on six Woods Hole region algal species. Additional algal hosts mentioned by Prenant and Teissier (1924, p. 23) are the Florideae, Himanthalia, and *Saccorhiza bulbosa.*

There is great variation in the appearance of the colonies. Their color ranges from white to reddish orange to a dull red. Some are smooth, flat, and shining, others rough and extended beyond the thalli. They may be lamellate, one colony growing over another. No description of the species is necessary because Figures 58 and 59 show the ovicells, and Figures 61 and 63 show the typical zooecial appearance, growth habit, aperture shape, and disposition of the avicularia. The avicularia grade in size (Figs. 57–60), but are always sharply pointed. Calcification may sometimes obliterate them and the aperture (Fig. 62).

In the Woods Hole area, larvae were found in ovicells in July and August (the times when collection was made) and undoubtedly occurred before and beyond these dates. At Beaufort, North Carolina, they are found the year round, according to McDougall. McDougall (1943, p. 340) observed that the times of greatest abundance of larvae (as judged by settlings on experimental substrata there at Beaufort) were in May, June, September, October and November.

Scruparia Ambigua

(Figures 64–65)

Most of the specimens of this dainty little dendritic form were dredged off Gay Head, Martha's Vineyard, on VII–30–1946. Some were growing on *Bugula turrita,* others on eleven algal species. It also grew in close association with hydroids and *Hippothoa hyalina.*

Scruparia ambigua zoids are yellowish, horny, transparent, and slender. They ranged from 0.345 to 0.495 mm. in length, the average of 12 specimens being 0.431 mm. The branching of the colony is quite open. Tentacles numbered ten in each of two zoids. No ovicells or larvae were found in the present material. Barrois (1877, p. 194) found ovicells and larvae at Roskoff during the month of June.

Hastings (1941) made a careful study of this species and differentiated *Scruparia ambigua* from *S. chelata* on the basis of the opesial slant and encrusting zooecia. In *S. ambigua* the opesial rim is parallel to the basal wall of the zooecium, and the free zooecial branches arise from a series of encrusting zooecia, as in Figure 65.

Scruparia Clavata

(Figures 66–67)

A few scraps of this delicate, horny, dendritic, transparent bryozoan were growing on *Laminaria Agardhii* which was dredged off Gay Head, Martha's Vineyard on VII–30–1946. Some ovicells were present. The zooecia bearing them were slightly smaller (Fig. 67) than the other zooecia. The zooecial orifice is much smaller than that of *Scruparia ambigua*.

Marcus (1940, p. 208) created a new genus *Haplota* for *S. clavata*.

Smittina Trispinosa

(Figures 68–71)

This species was found with great frequency on shells and rocks, sometimes many layers in thickness on the latter. However, its occurrence on algae was infrequent. *Chondrus crispus* (from North Falmouth, Mass.), *Laminaria Agardhii*, *Phyllophora Brodiaei*, and *P. membranifolia* from Woods Hole had a few colonies.

Rock colonies or nodules often are a light mustard yellow color; colonies on algae, however, were never that striking a color, but were ivory or iridescent.

Colonies are very fine grained in general appearance. The species shows a great deal of variation, depending upon age, degree of calcification, and nature of the substratum.

Summary

1. A total of 30 bryozoan species was reported from 37 species of marine algae.

2. Five bryozoan species were reported from three species of green algae, 26 bryozoan species from 11 species of brown algae, and 27 bryozoan species from 23 species of red algae.

3. *Phyllophora membranifolia* yielded the greatest number of bryozoan species (23), *Chondrus crispus* and *Laminaria Agardhii* each yielded 20, *Phyllophora Brodiaei*, 15, and *Cystoclonium purpureum cirrhosum* yielded 11 bryozoan species.

4. Each of the three commonest bryozoa, *Aetea sica*, *Bowerbankia gracilis* and *Crisia eburnea*, was found on 18 algal species.

5. Each of the two next commonest bryozoa, *Electra pilosa* and *Hippothoa hyalina*, occurred on 17 red and brown algal species.

6. *Bugula turrita,* the next most common form, was found on 16 algal species.

7. *Crisia eburnea* and *Hippothoa hyalina* were common on over half the red algal species examined.

8. Some bryozoa seemed to grow most frequently and abundantly on certain algal species, namely:

a. *Alcyonidium polyoum* on *Chondrus crispus, Phyllophora Brodiaei,* and *P. membranifolia*
b. *Bowerbankia gracilis* on *Ascophyllum nodosum, Chondrus crispus, Fucus vesiculosus, F. vesiculosus spiralis, Phyllophora Brodiaei* and *P. membranifolia*
c. *Crisia eburnea* on *Chondrus crispus, Phyllophora Brodiaei* and *P. membranifolia*
d. *Electra pilosa* on *Laminaria Agardhii* and *Rhodymenia palmata*
e. *Flustrella hispida* on *Ascophyllum nodosum.*

9. To the 84 known Woods Hole region bryozoan species can be added three more: *Aetea sica, Cellepora dichotoma* and *Scruparia ambigua.* It is quite possible that some of the previously reported *Aetea anguina* and *Cellepora americana* material may have included *Aetea sica* and *Cellepora dichotoma,* respectively.

10. Algal collections from New Rochelle, N. Y., yielded some of the same bryozoan species as are found in Woods Hole, namely: *Alcyonidium polyoum, Bowerbankia imbricata, Cryptosula pallasiana, Electra hastingsae, Membranipora lacroixii*(?), and *Pedicellina cernua.*

11. Algal collections from Rye, N. H., yielded some of the same bryozoa as are found at Woods Hole, namely: *Callopora aurita, Cribrilina annulata, Cribrilina punctata, Crisia eburnea, Electra pilosa, Hippothoa hyalina* and also a form, *Lichenopora hispida,* which did not occur at Woods Hole.

12. Twenty-six of the thirty bryozoan species were carefully illustrated.

13. Tentacle number counts were made for 15 species.

14. Three bryozoan species were collected, observed, or known to be in the larva-producing stage on algae in late June; seven species in July; ten in August; two in September. These were chance observations and the number of species would have been greater if more exhaustive collections over a greater number of months could have been made.

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STRATIFICATION AND DEFORMATION OF ARBACIA PUNCTULATA EGGS CENTRIFUGED IN CAFFEINE SOLUTIONS

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Introduction

During an investigation of the effect of methylated purines upon cellular behavior, the following problems arose: Does contact with the trimethylated purine, caffeine, which is known to influence cellular metabolism, affect the viscosity of the cytoplasm of the cell; and is there any evidence that this alkaloid influences the forces at the cell surfaces? Heilbrunn's informative studies (1926, 1928, 1943) on viscosity and surface forces, with reference to numerous chemical substances, did not deal adequately either with alkaloids as a group, or with caffeine in particular. Centrifugation followed by a study of the degree of granule stratification within the cytoplasm, together with changes in form of the cell itself, offered a satisfactory method of approach to these questions. From the experiments described below, it will be seen that caffeine does not alter the viscosity of the unfertilized egg but acts upon the membrane and cortical tension forces, thereby influencing cleavage in the fertilized egg. In the higher concentrations, even sperm entry is prevented by caffeine.

The temperature factor is not significant in the work reported here, since caffeinized eggs were centrifuged at the same time and temperature as control eggs from the same female. In this way, relative viscosity effects could be observed.

Methods and Materials

The author (1945, 1946a, b, 1946) has demonstrated by studies on O_2 consumption and comparative sensitivity of developmental stages that caffeine retards cleavage in Arbacia. In the present study, the unfertilized and fertilized eggs [unfertilized in sea water (SW), unfertilized in 0.10 per cent caffeine-in-sea-water (CSW); fertilized, i.e. normal egg (N ♀) × normal sperm (N ♂), in sea water, and fertilized N ♀ × N ♂ in 0.10 per cent caffeine-in-sea-water] were centrifuged at 10,000 × g for five, seven, and twelve minutes, and also at 3000 × g, 40 minutes after fertilization or at the equivalent time interval after shedding in the case of unfertilized eggs. This 40 minute period was chosen because that is when the viscosity of the protoplasm approaches the increased state typical at the time of cleavage. Comparable series employing other concentrations were also run.

Experiments were conducted at the temperature of running sea water. The appearance of uncentrifuged and centrifuged caffeinized eggs was compared with photomicrographs and descriptions by E. B. Harvey (1940). Differences in the degree of stratification (compactness) of the pigment granules and vacuoles and the height of the hyaloplasm zone after centrifugation were noted as evidence of

relative viscosities. To avoid error due to a time variable caused by the return of granules by Brownian movement, all photomicrographic records were made ten minutes after centrifugation.

Results and Discussion

Cleavage abnormalities in eggs centrifuged in CSW were no greater than those observed in the same concentration of CSW without centrifugation. There was no evidence that caffeine induced any primary change in viscosity which would prevent cleavage. Clearly defined effects were reproducible and similar in both unfertilized and fertilized eggs, but those in the former were more convincing because the normal viscosity changes during mitosis made it impossible to assume that controls and experimentals would be in exactly the same state.

Bank (1932), using *Arbacia punctulata*, reported stratification within the unfertilized eggs without centrifugation if they were held in 1 per cent caffeine for 48 hours. This is not surprising since caffeine was shown by the author (1945, 1948) to retard the O_2 uptake of the Arbacia cell. The facility with which the egg contents stratify due to such a factor as O_2 uptake cannot be determined by centrifugation. In the experiments described in this paper, an indication of a surface effect was the fact that Arbacia eggs cannot be fertilized when immersed in 1 per cent CSW. Both the eggs and sperm, however, survive for a considerable period in 1 per cent CSW, and the eggs can be fertilized and undergo partial development if transferred to sea water. Therefore, this concentration of caffeine does not destroy the internal physiological potentialities of these gametes with respect to fertilization. Over as long a period as 48 hours, the physical effect noted by Bank can be understood on the basis of the biochemical inhibition of cellular respiration, and/or as a surface effect, without assuming a primary viscosity change due directly to caffeine.

Among the results observed in the present series, the delay of deformation, reduction in actual fragmentation, and the sharper margins of the layers (apparent under the earlier conditions of the experiments) were the most readily distinguishable and clearly associated phenomena. De Vries (1947), in his studies on viscosity and tension at the surface in eggs of the fresh water snail, *Limnaea stagnalis* L., based the interpretation of his results primarily on the occurrence of vacuoles and granules in the hyaloplasm zone, as well as on the height of this zone. He pointed out that the height of the hyaloplasm zone depends on both viscosity and the degree of stretching, i.e. tension at the surface. Therefore, it occurred to the present writer that the closer packing of the pigment granules might be attributable to the fact that the pigment in the spherical cell had a shorter distance to fall than the pigment in the uncaffeinized normal cell, which is always elongated by centrifugal force to the degree applied in the experiments up to this time. It seemed desirable to eliminate the effect of the stretching factor and resulting deformation in order to clarify the significance of the degree of stratification in interpreting viscosity changes. Accordingly, unfertilized and fertilized eggs, both control and experimental, were centrifuged at only $3000 \times g$ for intervals varying from one-half to three minutes. These shorter centrifugations at $3000 \times g$ did not change the shape of the cells in either the control or the

caffeinized eggs, but did allow stratification. Therefore, comparisons of stratification could be made without the deformation factor.

In the absence of internal viscosity changes, the increased force required to break caffeinized eggs indicates a surface effect. Harvey (1931) estimated that the centrifugal force necessary to pull the Arbacia egg into two halves indicates that tension at the surface for a 25 per cent increase in area is less than 0.2 dyne per cm. with considerable variation in eggs. At 10,000 × g for 12 minutes, a count of five fields of each of the experimental caffeine series showed the percentage of breaking in the unfertilized eggs to be as follows: Controls in SW were 100 per cent broken; eggs in 0.02 per cent CSW, 12 per cent broken; in 0.10 per cent CSW, 0.50 per cent were broken; and in the 2.0 per cent CSW, only 0.08 per cent were broken although slight elongation did occur.

The apparent absence of any significant osmotic change (Cheney, 1948) as well as of demonstrable viscosity changes in the internal protoplasm, together with the delay in deformation reported here, would indicate that caffeine may initiate a change in the surface of the cell. Such an effect might involve both the membrane and the cortical protoplasm, which Harvey and Shapiro (1941) demonstrated to possess a considerably higher viscosity than the interior protoplasm in the eggs of *Arbacia punctulata* and *Asterias forbesii*.

Summary *

1. Caffeine does not change the existing viscosity state of the egg.

2. Egg fragmentation, under centrifugation, decreases with increased caffeine concentration.

3. The "apparent" effect of greater stratification of the granules in Arbacia eggs centrifuged in caffeine does not occur if the centrifugal force to which the eggs are subjected is sufficient to produce sedimentation but insufficient to cause deformation.

4. Evidence indicates that the delay of deformation in the caffeinized eggs, centrifuged at 10,000 × g or less, may be due to the action of caffeine (trimethylated purine) upon the total tension forces at the surface areas of both unfertilized and fertilized Arbacia eggs.

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A MUCIN CLOT REACTION WITH SEA-URCHIN FERTILIZIN

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Introduction

Recent work on the fertilizins (the sperm-agglutinating constituents of egg water) of the eggs of sea-urchins and other animals has shown them to be of the nature of mucoproteins. Tyler and Fox (1939, 1940) showed that the fertilizins of Strongylocentrotus and of Megathura possess protein characteristics, but are of low nitrogen content. Similar evidence has been obtained with Arbacia fertilizin by Kuhn and Wallenfels (1940) and with Psammechinus fertilizin by Runnström, Tiselius, and Vasseur (1942). The latter workers also obtained a positive carbohydrate test. Tyler (1948) reported the presence of reducing sugars to the extent of about 15 per cent in hydrolyzed, purified preparations of Strongylocentrotus fertilizin and identified galactose as one of the constituents. As will be shown later in this paper, the present author has found hexosamine to be present in amounts equivalent to about 2 per cent of the original material. According to Runnström, Tiselius, and Vasseur (1942) and Tyler (1946) the sea-urchin fertilizins are of pronounced acidic character.

Acidic mucopolysaccharides are known (cf. Meyer, 1945, and Stacey, 1946) to co-precipitate with proteins upon acidification of the native fluid or of the neutral extracts in the form of "mucin clots," stringy or granular precipitates, depending upon the conditions of precipitation. This reaction is given, for example, by hyaluronic acid (Meyer and Palmer, 1936) and has been used in the assay of the enzyme hyaluronidase (McClean, 1943). It was of interest to determine whether or not fertilizin preparations would give the mucin clot reaction. As the work reported here shows, fertilizin preparations do give such a mucin clot reaction. A titration method, based upon this, was developed for these preparations, and comparisons made with their sperm-agglutinating activity in untreated condition, after dialysis, and after exposure to heat and to ultra-violet irradiation.

Materials and Methods

The sea-urchins *Lytechinus pictus, Strongylocentrotus purpuratus* and *S. franciscanus* were used in these experiments. Most of the work was done with *S. purpuratus.*

Two kinds of fertilizin preparations were employed. One, which will be termed "crude fertilizin," was prepared by acidifying a 20 per cent suspension of washed eggs to pH 3–3.5, removing the supernatant fluid after five or ten minutes and readjusting the pH of this solution to 7–7.5. The other, which will be termed "purified fertilizin," was further subjected to alkali precipitation, dialysis against 3.3 per cent acid saline (pH 3.5–4) and alcohol precipitation according to the method described by Tyler (1948). Material prepared in this manner has been

found to be electrophoretically homogeneous (Tyler, unpub.). For the various tests the solutions were made up in 3.3 per cent NaCl at a pH of about 7.

Sperm-agglutinating titer of the fertilizin preparations was determined using the drop method of preparing two-fold serial dilutions with sea water in Syracuse watch glasses. To two drops of each dilution of fertilizin solution one drop of a uniform sperm suspension, usually 1 per cent (calculated as 1 cc. dry sperm per 100 cc. sea water suspension), was added. The highest dilution in which agglutination is observable under the microscope gives the titer of the preparation.

Tests for univalent fertilizin were made according to the method described by Tyler (1941) and Metz (1942). Essentially, this method consists of first treating sperm with the solution containing univalent fertilizin, which does not agglutinate the sperm, and then adding an equal volume of strong normal fertilizin solution to the suspension of sperm. Failure of the sperm to be agglutinated by the normal fertilizin presumably indicates that the combining groups on the sperm surface have been occupied by univalent fertilizin groups and are no longer available to unite with the normal fertilizin. Univalent fertilizin was obtained by heating and by irradiation with ultra-violet light of normal fertilizin preparations (cf. Tyler, 1941, and Metz, 1942).

Bovine serum albumin prepared by the Armour laboratories was used in 1 per cent solution for the co-precipitation tests.

Hyaluronic acid was obtained from human umbilical cords according to the method described by McClean (1943), whereby the distilled water extract of acetone-dried, ground cords, extracted with 90 per cent acetic acid according to the method of Meyer and Palmer (1936), was precipitated with 1.25 volumes of cold, potassium acetate-saturated 95 per cent alcohol. The precipitate was washed with alcohol, acetone, and ether and dried over P_2O_5. The dry product was dissolved in distilled water as required; a solution of 0.1–0.2 per cent of the dry material was clear, viscous and did not form a precipitate upon the addition of acetic acid, but co-precipitated with serum albumin in the presence of acetic acid, forming a stringy clot. In higher dilutions the mixture of serum albumin, acetic acid and hyaluronic acid solution resulted in the formation of a fine precipitate or turbidity. The highest dilution in two-fold serial dilutions in which turbidity was perceptible by visual inspection was taken as the titer of the hyaluronic acid solution.[1]

Experiments and Observations

Co-precipitation of fertilizin with serum albumin in acid solution

A viscous solution of crude fertilizin of *Lytechinus pictus* was prepared as described above and combined with a 1 per cent solution of bovine serum albumin in 0.9 per cent NaCl and 2 N acetic acid according to the method described by McClean (1943) in the mucin clot test. With this solution a very large clot was formed similar in character and appearance to the clot formed by hyaluronic acid. Purified fertilizin preparations of *Strongylocentrotus purpuratus, S. franciscanus,* and *L. pictus* were tested in the same manner and in each case a clot or precipi-

[1] A quantity of pure potassium hyaluronate was later supplied to me by the Schering Corporation, through the courtesy of Dr. W. Alan Wright and Dr. Erwin Schwenk.

tate formed, depending upon the concentration of the solution. In Figure 1 a series of photographs of the mucin clot reaction of *S. purpuratus* fertilizin is shown, with the reaction given by hyaluronic acid for comparison.

By taking advantage of the biological activity of fertilizin, i.e., its sperm-agglutinating activity, a simple test was performed which provided definitive evidence that it is the fertilizin which is co-precipitated with serum albumin and not some hitherto undetected component of the material.

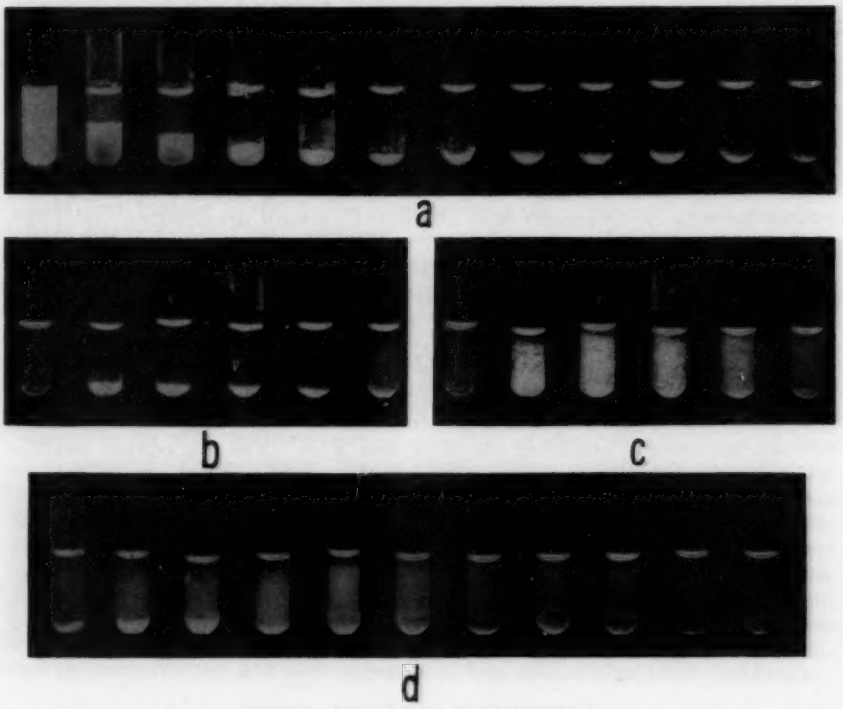

FIGURE 1. Mucin clot reactions of *Strongylocentrotus purpuratus* fertilizin and of hyaluronic acid in two-fold dilutions. **a.** Purified *S. purpuratus* fertilizin. The first tube contains fertilizin solution and serum albumin, but distilled water instead of acetic acid. The opacity of the first tube is due to the opalescence of the mixture. **b.** Crude *S. purpuratus* fertilizin. The first tube contains fertilizin solution and acetic acid but 0.9 per cent saline instead of serum albumin. **c.** The same as **b** with the tubes shaken prior to being photographed. **d.** Hyaluronic acid.

A solution of purified fertilizin in 3.3 per cent saline was mixed with serum albumin and the pH brought to about 3.5 with 2 N acetic acid. The resulting precipitate was thrown down by centrifugation, the supernatant withdrawn and its pH adjusted to 7. As shown in Table I, the supernatant exhibited no sperm-agglutinating activity.

TABLE I

Sperm-agglutinating activity of the supernatant and of the precipitate recovered separately after the addition of bovine serum albumin to an acidified solution of purified fertilizin, and results of control tests

Reaction mixture		Per cent of sperm-agglutinating activity*
0.5 ml. fertilizin in 3.3% saline	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	Supernatant: 0 Dissolved precipitate: 100
	0.05 ml. distilled H_2O 0.2 ml. 1% bovine serum albumin in 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 0.9% saline	100
	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline, acidified and neutralized	100
0.5 ml. 3.3% saline	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	0

* Sperm-agglutinating activity of fertilizin, distilled water and 0.9% saline mixture taken as 100%.

It had previously been found that the precipitate formed by the addition of serum albumin to a fertilizin solution acidified to a pH of about 3.5 dissolves completely at a pH of 5.6 or higher. After centrifugation and withdrawal of the supernatant of the material being tested, the precipitate was resuspended in 3.3 per cent NaCl solution, its pH brought to about 7, and the volume made equal to that of the original mixture. Upon testing this solution it was found, as is shown in the table, that the sperm-agglutinating activity was equal to that of the original fertilizin solution, showing that the activity was recovered quantitatively from the precipitate.

A number of control tests were made, the results of which are summarized in Table I. These showed that (1) co-precipitation of fertilizin and serum albumin does not occur in the absence of acid; (2) it does not occur in acidified solution in the absence of added protein; (3) the presence of albumin does not affect the sperm-agglutinating activity of the fertilizin; (4) the sperm-agglutinating activity is not affected by acidification and subsequent neutralization of the solution, and (5) sperm agglutination does not occur in saline solution in the absence of fertilizin, nor is a precipitate formed when albumin and acid are added to saline.

Quantitative recovery of the sperm-agglutinating activity from co-precipitated fertilizin and serum albumin, which is achieved by the simple expedient of raising the pH to 5.6 or higher, shows that the specific combining groups of the fertilizin are not irreversibly altered by its reaction with the albumin. The significance of this phenomenon cannot be evaluated at the present time, however, since essentially nothing is as yet known of the structure of the fertilizin molecule.

Titration by the mucin clot method

The method employed for titration was as follows: Two-fold serial dilutions of fertilizin in 3.3 per cent NaCl at about pH 7 are made in 10 × 75 mm. test tubes in 0.5 ml. quantities. To each tube 0.05 ml. of 2 N acetic acid is added and the contents mixed. The tubes are inclined and 0.2 ml. of 1 per cent bovine serum albumin in 0.9 per cent saline is slowly pipetted down the side of each tube; after this the tubes are carefully returned to a vertical position and in most cases a ring of precipitate forms immediately at the zone of contact between the albumin solution and the acidified fertilizin solution. The rings are easily detected at high dilutions in which a diffuse turbidity is difficult to detect and score visually. The reactions are read at once in good natural light, since the rings tend to disperse rapidly as the albumin diffuses through the mixture. The highest dilution at which a ring is observed is taken as the mucin clot titer of the preparation.

Effect of pH, albumin concentration and salt concentration

Numerous titrations with the same stock samples of purified fertilizin using the method described above have given consistently identical mucin clot titers in the course of routine testing. It was thought advisable, however, to carry out some controlled tests to determine in a more definitive manner the amount of variability in titer to be expected within a rather limited range of pH, albumin concentration, and salt concentration.

A sample of a purified fertilizin solution from eggs of *S. purpuratus* was centrifuged at 3000 r.p.m. for five minutes. A slight sediment was thrown down, the clarified supernatant was drawn off, and the pH adjusted to 7.0 with the glass electrode. To assure maximum uniformity of different samples of the supernatant, it was thoroughly mixed before removing an aliquot. Two-fold dilutions were made in 0.5 ml. quantities with 3.3 per cent NaCl solution. Serum albumin was made up in 1 per cent solutions, and the same stock solution of 2 N acetic acid was used throughout. Various amounts of the latter two solutions were added to different sets of tubes of the fertilizin dilutions. The total albumin and salt concentration in the different sets was thus altered, as noted below. The pH was measured with a Beckman glass electrode pH meter. After each titration the pH of the final mixtures was measured in the first tube, in the tube giving the end-point, and in an intermediate tube. The maximum difference in pH observed within any set was 0.26 unit. The results obtained in a series of nine titrations are given in Table II. Each of the pH values listed represents the mean of three determinations in each set. The total salt concentration is expressed as per cent NaCl. The mucin clot titers (last column of table) are the end points of visible precipitation as determined by the ring method described above.

As the data in the table show, no marked difference in mucin clot titer occurs, for the most part, as a result of the differences in pH, total salt and albumin concentrations employed. In titration 5, the observable end-point would probably have been at least one dilution higher had rings formed. The failure of rings to form is attributable to the relatively large quantity of serum albumin solution used; as noted above, in high dilutions a diffuse turbidity such as was produced in this case is more difficult to detect visually than a ring at the same dilution. It has

been found that 0.5 ml. of albumin solution is about the largest quantity practicable for obtaining consistent ring formation. Since there is always a tendency to pipette too rapidly when a large number of tests is being performed, it has proven more convenient to use 0.2 ml. of albumin solution.

The evidence presented in Table II indicates that pH is not a critical factor with respect to observed titer within the range tested. The albumin concentration is not critical, nor is the total salt concentration of the system in the range from 2.0 per cent to 3.1 per cent. Below 2.0 per cent the salt concentration may be a more important factor, as shown by the higher titer obtained in titration 6. Although the total salt concentration in titration 5 is the same as that in number 6 (1.6 per cent), the two sets are not comparable for the reason mentioned above.

TABLE II

Mean pH, total albumin concentration, total salt concentration and mucin clot titers in nine titrations using uniform samples of a homogeneous purified fertilizin preparation of S. purpuratus

Titration no.	Ml. 2 N acetic acid	Ml. 1% albumin solution	NaCl concentration of albumin solution, per cent	Mean pH	Total albumin concentration, per cent	Total NaCl concentration, per cent	Mucin clot titer
1	0.05	0.1	0.9	3.09	0.15	2.7	64
2	0.05	0.2	0.9	3.30	0.27	2.4	64
3	0.05	0.3	0.9	3.33	0.35	2.3	64
4	0.05	0.5	0.9	3.54	0.47	2.0	64
5	0.05	1.0	0.9	3.74	0.65	1.6	32*
6	0.05	0.5	0.0	3.58	0.47	1.6	128
7	0.05	0.5	3.3	3.58	0.47	3.1	64
8	0.01	0.2	0.9	3.88	0.28	2.6	64
9	0.02	0.2	0.9	3.78	0.28	2.5	64

* Scored as turbidity, not as ring.

In the next section it will be shown that the mucin clot and sperm-agglutinating titers of a fertilizin solution are both reduced after dialysis of the preparation against distilled water with consequent removal of salt (NaCl). The data to be presented indicate that the physical state of the fertilizin is reversibly modified in the absence of electrolytes, at least under certain conditions of temperature. It seems likely, therefore, that there may exist some optimum concentration of electrolytes between zero concentration and that represented by 2.0 per cent NaCl (equivalent to an ionic strength of 0.34) at which co-precipitation of fertilizin and serum albumin attains a maximum. Further analysis of the effect of salt concentration on the co-precipitation of fertilizin and serum albumin is under way and will be reported in a later communication.

In the system as employed in the routine titration procedure in the present investigation, however, the mean pH, total albumin and total salt concentration fall well within the range for each of these factors in which identical titers are obtained, using uniform samples of a homogeneous fertilizin preparation.

Mucin clot and sperm-agglutinating titrations of fertilizin in salt-free solution

A sample (I) of a stock preparation of purified fertilizin in 3.3 per cent saline was made salt-free by dialyzing against distilled water at 1° C. until it no longer formed a precipitate with $AgNO_3$. It was found that a white, flocculent material was present in the dialyzed solution, whereas no such flocculence was evident in the control in 3.3 per cent NaCl solution which was kept at 1° for the same period. A considerable reduction in both mucin clot and sperm-agglutinating titer of the salt-free sample as compared with the control was observed. In the mucin clot titration, the character of the precipitate formed in the salt-free preparation upon the addition of serum albumin in the presence of acid differed from that in the control in being of larger particle size and somewhat stringy. Difficulty was encountered in making sperm-agglutinating titrations (where the salt content of the preparation was adjusted just prior to titration to 3.3 per cent NaCl by adding an equal volume either of 6.6 per cent NaCl or of double sea water to the salt-free solution) in cases where suspended material was present. The spermatozoa clumped about the particulate matter, and it was impossible to score the dilutions satisfactorily. Where tests were made with samples of the dialyzed solution in which the amount of suspended material was visibly less than was originally present, both the mucin clot precipitation and sperm agglutination occurred in the manner characteristic of the control solutions; in these cases the two titers were also somewhat higher, although they did not necessarily equal the values obtained for the control solution.

A second sample (II) of the same stock preparation of purified fertilizin was dialyzed against distilled water until salt-free. This time the first five changes of the water used for dialysis, totaling 3 liters, were saved, combined, and lyophilized to dryness. The residue was taken up in about 10 cc. of distilled water and the resulting solution was approximately isotonic with sea water, as shown by the fact that sperm of *S. purpuratus* remained active when placed in it. This "dialysate-concentrate" was 300 times more concentrated than the original dialysate, but proved to be negative for both mucin clot formation and sperm-agglutinating activity. Sample II behaved in all respects like sample I. The results of tests with the two samples are summarized in Table III.

The evidence obtained from the present experiments indicates that at temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes. Macroscopic aggregates may appear in a fertilizin preparation under these conditions, and there is a correlated decrease in the mucin clot and sperm-agglutinating titers. Under the influence of added salt and elevated temperature (up to 21.5° C.), either separately or combined, there occurs a correlated decrease in the amount of visible macroscopic material and increase in mucin clot and sperm-agglutinating titers to values approaching those obtained with control solutions. The negative results of tests with the "dialysate-concentrate" show that there was no actual loss of fertilizin during dialysis.

Analysis of a purified fertilizin preparation

The co-precipitation of fertilizin with protein in acidic solution in a manner analogous to the behavior of acid mucopolysaccharides suggests affinity of fertilizin with this class of substances. It has recently been claimed, moreover, that a preparation

TABLE III

Results of titrations of salt-free fertilizin preparations of S. purpuratus after various treatments

Test no.	Fertilizin preparation	Treatment after removal from dialysis bath at 1° C.	Sperm-agglutinating titration		Mucin clot titration	
			pH	Titer	pH	Titer
1	I	None			5.2	8
2	I	Salt content adjusted to 3.3% NaCl. pH adjusted	7.2	64		
3	I	Salt content adjusted to 3.3% NaCl			5.2	16
4	I	2.5 hrs. at 21.5° C.			5.2	16
5	I	2.5 hrs. at 21.5° C. pH adjusted			7.2	16
6	I	Dialyzed vs. 3.3% NaCl at 1° C. 2 hrs. at 21.5° C. pH adjusted	7.0	64	7.0	16
7	I	Dialyzed vs. 3.3% NaCl at 1° C. 24 hrs. at 8° C. pH adjusted	7.45	1024	7.45	32
8	I	Control. Not dialyzed vs. distilled water. pH adjusted	7.4	512	7.4	64
9	II	1 hr. at 21.5° C. pH adjusted			6.9	32
10	II	Salt content adjusted to 3.3% NaCl. 3 hrs. at 21.5° C. pH adjusted	6.9	512		
11	II	Salt content adjusted to 3.3% NaCl. 1 hr. at 21.5° C. pH adjusted			6.9	64
12	II	Control. Not dialyzed against distilled water. pH adjusted	7.3	1024	7.3	128

from bull testes, presumably containing the enzyme hyaluronidase, is capable of causing the jelly of intact sea-urchin eggs to swell (Ruffo and Monroy, 1946; Monroy and Ruffo, 1947). It was of interest, then, to attempt to determine the extent to which fertilizin may be chemically similar to hyaluronic acid. The few available data, which have been reviewed briefly in the introductory section, indicate that fertilizin is by no means identical with hyaluronic acid. The present investigation has shown that fertilizin differs from hyaluronic acid to a marked degree with respect to the two chief constituents of the latter substance, hexosamine and glucuronic acid.

A homogeneous sample of a purified fertilizin preparation in 3.3 per cent NaCl was prepared as described above. In the present instance the supernatant fluid was filtered through hardened filter paper. The pH of the filtrate was adjusted to 7 and

TABLE IV

Results of chemical analysis of a purified fertilizin preparation of S. purpuratus

Filtrate					Hydrolysate	
Sperm-agglutinating titer	Mucin clot titer	Dry weight, mg./ml.	Total nitrogen, per cent	Glucuronic acid	Hexosamine, per cent	α-amino acids
2048	512	8.95	4.1	none	1.6	positive Ninhydrin

mucin clot and sperm-agglutinating titers were obtained. Aliquots of the filtrate were taken for the various analyses, which included determinations of dry weight, total nitrogen, hexosamine, α-amino acids and glucuronic acid. The results of the analyses are presented in Table IV. Dry weight per ml. was calculated from the weight of material precipitated from an aliquot of the filtrate with 1.25 volumes of cold 95 per cent alcohol. According to Tyler (1948), precipitation of fertilizin is complete under these conditions. The precipitate was washed with alcohol and dried in an oven at 55° C. to constant weight. Total nitrogen was determined for duplicate samples of the dried precipitate by the micro-Kjeldahl method. Another portion of the dried material was hydrolyzed by boiling in a sealed tube with 4N HCl for eight hours. Hexosamine was determined in an aliquot of the hydrolysate by the method of Palmer, Smyth and Meyer (1937). Another portion of the hydrolysate was treated with Ninhydrin reagent for the determination of α-amino acids. For the determination of glucuronic acid the colorimetric method recently described by Dische (1947b) was employed, using a sample of the original filtrate,

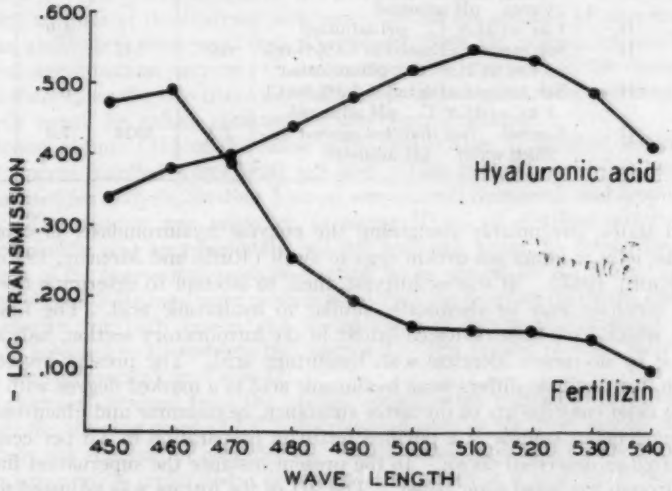

FIGURE 2. Absorption curves of reaction mixtures of mannose-thioglycolic acid-fertilizin and mannose-thioglycolic acid-hyaluronic acid. Wave lengths in mμ. Fertilizin $\Delta E_{(510-480)} = -0.097$; hyaluronic acid $\Delta E_{(510-480)} = +0.115$.

and for comparative purposes, a solution of pure potassium hyaluronate (Schering) was tested at the same time by the same method. According to Dische, the reaction of carbohydrates with -SH compounds in H_2SO_4, which differentiates between various classes of carbohydrates and individual hexoses and hexuronic acids, is highly characteristic for glucuronic acid when mannose is employed. This reaction is the basis of the test. The reaction mixture with glucuronic acid gives a typical absorption curve in the range 450–540 mμ, and it was found by Dische that the curve for hyaluronic acid is almost identical with that of glucuronic acid. In practice, accord-

ing to Dische, it is only necessary to measure the intensity of the mannose reaction at 510 and 480 mμ and subtract the second value from the first. This difference is positive for glucuronic acid and polyglucuronides, and negative for the other hexuronic acids. Figure 2 shows the absorption curves for fertilizin and for hyaluronic acid. The difference between the intensity of the mannose reaction with fertilizin at 510 and that at 480 mμ is negative, and hence it may be concluded that fertilizin does not contain glucuronic acid. This result is in agreement with previous results obtained by Tyler (unpub.) using an earlier method of Dische's (1947a). As shown in Table IV, fertilizin does contain hexosamine, but in small amount, which is in agreement with earlier results obtained by the present author using Kunitz's (1939) method. The Ninhydrin reaction was very weak but probably positive. Total nitrogen (4.1 per cent) of this material is somewhat lower than has been reported previously for *S. purpuratus* fertilizin by Tyler and Fox (1940), who found an average total nitrogen content of 5.2 per cent with crude preparations.

These data show that fertilizin differs markedly from hyaluronic acid in its chemical constitution. It is obvious, therefore, that the ability to give the mucin clot reaction does not by any means indicate close similarity between fertilizin and hyaluronic acid, even though it may be evidence that the former is related to the group of acidic mucopolysaccharides.

At the suggestion of Dr. Albert Tyler, the mucin clot titration procedure was used in conjunction with sperm-agglutinating titrations to investigate the effects of various kinds of treatment on fertilizin. In the following sections the results of parallel titrations of preparations subjected to heat and to ultra-violet irradiation are presented.

Parallel titrations with heat-treated fertilizin solutions

According to Tyler and Fox (1940), the sperm-agglutinating activity of *Strongylocentrotus purpuratus* fertilizin is rapidly destroyed at 100° C. The rate of inactivation, according to these authors, is a function of the pH, the fertilizin being most stable in the range from 4 to 7. Their data show that at pH 7.3 the agglutinin is 95–100 per cent inactivated in 20–30 minutes at 100°. In the present experiments in which *S. purpuratus* purified fertilizin solutions were used, the preparations have proven to be considerably more heat-stable than the material used by Tyler and Fox. Since the solutions employed by Tyler and Fox corresponded to crude fertilizin as defined in this paper, it may well be that the relatively purer condition of the fertilizin in the present preparations accounts for its greater stability.

Initial loss of agglutinating activity does not appear to involve complete destruction of fertilizin. At first the agglutinating fertilizin is converted into a "univalent," non-agglutinating form (cf. Tyler, 1941, Metz, 1942). It was of interest, accordingly, to test samples of heat-treated fertilizin for their univalence (inhibition) titer as well as for their sperm-agglutinating and mucin clot titers. The method employed for detecting univalent fertilizin has been briefly described in an earlier section of this paper; determination of inhibition titer consists in determining the greatest dilution in which no agglutination occurs upon the addition to the test dilutions of equal amounts of normal (untreated) fertilizin solution (Metz, 1942).

In the first experiments 1.5 ml. samples of the stock purified fertilizin solutions were placed in 13 × 100 mm. test tubes and immersed in a boiling water bath. The

exposed portions of the tubes were cooled by means of a stream of air so that heating could be continued for long periods without appreciable loss of fluid. Since sperm are quickly inactivated in even slightly hypertonic medium, the fact that the sperm remained active in the solutions that had been heated was assumed to indicate that evaporation of water from the tubes during heating was insignificant. A thermometer placed in the water bath with the tubes showed that the temperature of the bath fluctuated between 96° and 98° C.

In later experiments a quantity of fertilizin solution large enough to permit the withdrawal of a number of 1.5 ml. samples was placed in a flask with a reflux condenser attached by means of a ground glass joint. The solution was refluxed and loss of water was thus kept to a minimum. The temperature of the boiling fluid in the flask could be assumed to be about 100° C. A considerable excess of solution was used so that its concentration would not be significantly affected by the slight amount of water that failed to run back down. Before withdrawing a sample, the neck of the flask and the lower part of the condenser were cooled with cold water from a wash bottle. In all of the experiments the pH of the fertilizin solutions was adjusted with the glass electrode just before heating was begun; a control sample was allowed to stand at room temperature throughout the total time of heating. As each sample was removed from the water bath or from the reflux flask,

TABLE V

Results of parallel titrations of heat-treated, purified fertilizin preparations of S. purpuratus

Fertilizin preparation	Initial pH	Sample	Temperature, degrees Cent.	Time in hours	Final pH	Titer		
						Sperm agglutination	Mucin clot	Inhibition
I*	7.1	a	96–98	0.5	—**	256	64	—**
		b	96–98	1.0	—	64	64	—
		Control	room	1.0	—	512	64	—
II*	7.85	a	96–98	4.5	—	16	64	—
		Control	room	4.5	—	256	64	—
III*	7.4	a	96–98	3.5	—	128	256	0
		b	96–98	5.5	—	64	64	32
		Control	room	5.5	—	1024	256	—
IV***	7.5	a	100	2.0	7.5	4096	512	—
		b	100	3.0	7.2	4096	512	—
		c	100	5.0	7.1	2048	256	—
		d	100	6.0	7.1	2048	256	0
		e	100	7.5	7.1	1024–2048****	256	0
		Control	room	7.5	6.8	4096	512	—

* Individual 1.5 ml. samples heated in water bath.

** Dashes indicate not tested. Zero inhibition titer indicates tested but inhibition not detected.

*** Samples (1.5 ml.) withdrawn from refluxed solution.

**** A trace reaction probably present in the higher dilution.

it was placed immediately in the freezer. In the later experiments the pH of each heated sample was recorded before it was frozen. The titrations were performed as soon thereafter as possible. The results of the experiments are presented in Table V.

As may be seen from the table, reduction of sperm-agglutinating titer by heating is not necessarily accompanied by parallel reduction in mucin clot titer. Thus, for example, samples IIa and IIIa, heated for 4.5 and 3.5 hours respectively, showed no significant reduction in mucin clot titer although the sperm-agglutinating titer of the former was reduced to about 6 per cent and that of the latter to about 12 per cent of the original values. Sample IIIb was heated for 5.5 hours with a reduction of sperm-agglutinating titer to approximately 6 per cent of its original value. In this case the mucin clot titer was reduced to 25 per cent of the original value. Samples IVa–IVe show a more nearly parallel reduction of sperm-agglutinating and mucin clot titers than any of the others. Preparation IV was refluxed. After boiling for 7.5 hours the sperm-agglutinating titer was reduced to 25–50 per cent of the original value and the mucin clot titer was reduced to about the same per cent of the original value. Sample IIIb was the only one which gave an inhibition (univalence) titer. In the samples which were tested for inhibition but in which none was detected (IIIa, IVd, IVe), it is probable that insufficient univalent fertilizin was present in the high dilutions to permit detection. The inhibition test is unambiguous only in dilutions containing sufficient univalent fertilizin to react with most of the added sperm. In the high dilutions enough sperm remain uncombined to be agglutinated upon the addition of normal fertilizin and thus obscure the slight amount of inhibition that may be present. In the present experiments, sample IIIb was the only one in which sufficient univalent fertilizin was produced in the lower dilutions to give clear-cut evidence of inhibition. Since the inhibition titer of IIIb was 32, while the mucin clot titer was 64, it appears that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. Stronger evidence to support this view was afforded by experiments in which fertilizin was irradiated with ultra-violet light.

Parallel titrations with ultra-violet irradiated purified fertilizin preparations

Metz (1942) showed that univalent fertilizin is produced by irradiation of normal (multivalent) fertilizin by ultra-violet rays. In the present experiments ultra-violet irradiation was carried out in an apparatus consisting of glass tubing, 150 × 35 mm., fitted on the mid-section of a 15 watt General Electric "Germicidal" lamp, the diameter of which is 25 mm. The major part of the output of this lamp is concentrated in the 2537 Å wave-length band. The space between the outer wall of the lamp and the inner wall of the tubing is the irradiation chamber. The chamber and lamp assembly is mounted on a motor-driven rocker. An opening in the top of the chamber, which can be closed with a rubber stopper, permits the introduction and withdrawal of fluid. The chamber is cooled by means of a small electric fan mounted on the rocker platform; when the fan is in operation the temperature of fluid inside the chamber does not rise above 35° C. during irradiation.

In the first experiment, a purified fertilizin preparation of *S. purpuratus*, the pH of which was first adjusted to 7, was irradiated for a total of 2.5 hours. It was found, as shown in Table VI, that the sperm-agglutinating titer was reduced to

TABLE VI

Results of ultra-violet irradiation of purified fertilizin

Fertilizin preparation	Sample no.	Initial pH	Time of irradiation in hours	Final pH	Sperm agglutination titer	Mucin clot titer	Inhibition titer
I	Control	7.0	—	—	256	64	—
	1	7.0	2½	—	4	32	—*
II	Control	7.67	—	6.60	1024	1024	—
	1	7.67	4½	5.52	0	1024	128
	2	7.67	6	5.49	0	1024	128
	3	7.67	7½	5.49	0	512	4
	4	7.67	9	5.49	0	256	4

* Univalence present in this sample by inhibition test but titer not obtained.

about 2 per cent of the original value and the mucin clot titer was decreased to 50 per cent of the original. Tested by the inhibition method, the irradiated preparation was found to contain univalent fertilizin. The inhibition titer of this sample was not obtained. In a second experiment, a quantity of the fertilizin preparation which was found to be very heat-stable with respect to its sperm-agglutinating activity (preparation IV of the preceding section) was irradiated. The pH of the solution was first adjusted to 7.7. Small portions (1.5 ml.) were withdrawn at intervals up to nine hours; the first sample was removed after 4.3 hours of irradiation. A control sample was allowed to stand in natural light (filtered through window glass) at room temperature throughout the entire period of irradiation. Immediately upon the removal of each sample from the irradiation chamber, its pH was measured with the glass electrode, and then it was placed in the freezer. All of the samples, including the control, were stored in the freezer until the titrations could be performed. As shown in Table VI, all of the irradiated samples showed complete loss of agglutinating activity. Tested by the inhibition method, all of them were found to contain univalent fertilizin. The inhibition titers showed a progressive decrease as time of irradiation was increased. The mucin clot titers also showed a progressive decrease with increased time of irradiation. After nine hours the mucin clot titer was reduced to 25 per cent of its original value, and the inhibition titer was reduced to about 6 per cent of the value found after 4.3 hours of irradiation.

The results of these experiments demonstrate conclusively that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. They also show that ultra-violet irradiation is a more effective agent than heat in converting multivalent, purified fertilizin to the univalent condition. The progressive decrease in inhibition titer found in the second experiment indicates that degradation of the fertilizin by ultra-violet light proceeds beyond the stage in which it exhibits univalence.

DISCUSSION

In general it may be said that the mucin clot titer of untreated fertilizin preparations parallels their sperm-agglutinating activity. Sperm agglutination is usually detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

Destruction of the sperm-agglutinating activity of fertilizin is not necessarily accompanied by a reduction of mucin clot titer. Conversely, however, it is clear that fertilizin which has been subjected to treatment that causes a reduction in mucin clot titer, for example heating or irradiation by ultra-violet light for extended periods, will invariably show at least a parallel decrease in sperm-agglutinating activity. It has been shown in the experiments with ultra-violet irradiation that the capacity of the fertilizin to agglutinate sperm may be completely destroyed with but little, if any, loss of its ability to give the mucin clot reaction. The evidence shows that when the agglutinating (multivalent) form is degraded to the non-agglutinating (univalent) form, the latter continues to co-precipitate with protein in the mucin clot reaction. If a preparation in which all of the fertilizin has been made univalent is subjected to continued irradiation by ultra-violet light, a progressive decrease in both mucin clot and inhibition titers occurs.

The phenomenon of sperm agglutination by fertlizin has been interpreted by Tyler (1941, 1942, 1947, 1948) as an antigen-antibody type of reaction in which complementary combining groups of a substance (antifertilizin) on the surface of the sperm cells unite in "lock and key" fashion with the combining groups of fertilizin. Where a number of such combining groups are available on the surface of the fertilizin molecule, agglutination occurs as the result of the building up of a lattice, as postulated for analogous immunological reactions by Heidelberger (1938) and Marrack (1938). The formation of univalent fertilizin is brought about by various agents—e.g. heat, ultra-violet light, x-rays—which, according to Tyler (1941), split the molecule into fragments, each of which contains a single combining group. These fragments are still of large size, since they are non-dialyzable (Tyler, 1941). They are also capable of co-precipitating with protein in the presence of acid, giving the mucin clot reaction.

The ability to give the mucin clot reaction is, at least in the case of hyaluronic acid, presumably a function of the degree of polymerization of the molecule (Meyer, 1947). Depolymerized molecules are incapable of giving the reaction. Although fertilizin has been shown to be very different from hyaluronic acid in its chemical composition, the fact that it co-precipitates with protein in acid solution in an analogous manner suggests that it may be similar in its physical structure. Thus, fertilizin may normally exist in a polymerized condition. Sperm-agglutinating activity may, then, accompany a range of polymer size, and the univalent condition may represent a state of polymerization with which but a single combining element is associated. Degradation of multivalent fertilizin to the univalent form would then entail a progressive splitting off of relatively stable univalent units. The evidence from the experiments with ultra-violet irradiation indicates that the univalent form is in fact the more stable, since complete conversion to univalence was observed after 4.3 hours of irradiation, whereas even after nine hours both the inhibition and mucin clot titers retained significant values.

Summary

1. Preparations of fertilizin of three species of sea-urchin have been found to give a mucin clot reaction similar to that given by hyaluronic acid. Upon the addition of bovine serum albumin to an acidified solution of fertilizin, a precipitate forms which dissolves at a pH of 5.6 or higher. All of the sperm-agglutinating activity accom-

panies the precipitate and it is recovered quantitatively when the precipitate is dissolved.

2. A method for the determination of mucin clot titer of fertilizin is described.

3. At temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes by dialysis. Macroscopic aggregates appear, accompanied by a parallel decrease in mucin clot and sperm-agglutinating titers. Disappearance of the aggregates is accompanied by an increase in both titers.

4. Chemical analysis of fertilizin shows that it contains no glucuronic acid, about 2 per cent hexosamine and amino acids. Fertilizin, therefore, differs greatly from hyaluronic acid, but its ability to give the mucin clot reaction suggests an affinity with the class of mucopolysaccharides.

5. In general, mucin clot titer parallels sperm-agglutinating titer of the same untreated fertilizin preparation, although sperm agglutination is detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

6. Parallel mucin clot and sperm-agglutinating titrations were made with fertilizin preparations in untreated condition and after exposure to heat and to ultra-violet irradiation. The purified preparations used in these experiments proved to be exceptionally heat-stable; irradiation by ultra-violet light was found to be a more effective treatment in degrading the material.

7. Destruction of the sperm-agglutinating activity of fertilizin by heat and by ultra-violet irradiation does not necessarily cause a parallel decrease in mucin clot titer. The mucin clot reaction continues to be given by preparations in which all of the fertilizin has been converted from the normal, agglutinating condition to the non-agglutinating, "univalent" form. Continued irradiation of the univalent fertilizin is accompanied by a progressive decrease in both inhibition and mucin clot titer.

8. It is suggested that fertilizin may normally exist in a polymerized condition and that the non-agglutinating, "univalent" condition may represent a relatively more stable lower polymer of the native, agglutinating form.

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THE ANAPHASE MOVEMENT OF CHROMOSOMES IN THE SPERMATOCYTES OF THE GRASSHOPPER

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Among the many complex processes involved in the division of cells, the movement of the chromosomes at anaphase is most accessible to a causal analysis. The beautiful preciseness of the processes involved in the orderly separation of chromosomes has for a long time enticed biologists to search for their physico-chemical basis. However, before an analysis on this level is possible, it is necessary, first, to know what structural differentiations of the cell are involved, and secondly, to have detailed quantitative descriptions of the processes based on a study of living cells.

A previous analysis of chromosome movement in certain insects (Homoptera and Hemiptera) has shown that the structures involved in anaphase movement are the kinetochores on the chromosomes, the chromosomal fibers, which connect the kinetochores to the spindle, and the spindle body (Ris, 1943). The kinetochore determines the nature of the chromosomal fibers, which in the case of these insects are broad and sheet-like and attached to the entire length of the chromosome (cf. Hughes-Schrader and Ris, 1941). The movement of the chromosomes consists of two separate processes: first, the shortening of the chromosomal fibers, which moves the chromosomes to the poles of the spindle; and secondly, the elongation of the spindle body, which further separates the chromosomes. In the Homoptera and Hemiptera these two components of anaphase movement are separated in time, so that first the chromosomes move to the poles of the spindle and then, after a pause of a few minutes, the spindle body stretches and carries the chromosomes further apart.

In most animals and plants the chromosomal fibers are narrow bundles attached to a definite, restricted region of the chromosome. In this paper the spermatocyte divisions of the grasshopper were chosen in order to analyze chromosome movement in an organism with localized kinetochore.

Material and Methods

The measurements recorded here were made on spermatocytes of *Chorthophaga viridifasciata*. A few measurements on *Dissosteira carolina, Melanoplus femur-rubrum, Arphia xanthoptera* and *Hippiscus spec.* gave similar results.

The spermatocytes of the grasshopper are classical material in the study of living cells in division (Chambers, 1914, 1924; Lewis and Robertson, 1916; Belar, 1929; Baumgartner and Payne, 1931). The usual technique consisted in breaking the testis follicles and spreading the cells on a coverglass in Locke's or Ringer's solution. Baumgartner and Payne (1931) showed that the follicles can be left intact and the cells studied with high powers. They pulled the testis through an opening

[1] Part of the work for this paper was done in the Department of Biology, Johns Hopkins University.

of the body wall into a little pool of salt solution, but left it attached to the vasa efferentia. Since they had to remove the follicular membrane which contains the trachae, the testes may as well be completely removed from the animal. In the present work the testes were dissected out, the follicular membrane removed, and the follicles spread intact on a coverglass into a drop of Belar's solution (Belar, 1929). The coverslip was inverted over a depression slide and sealed with paraffin. Aseptic technique was not attempted since only preparations made on the same day were used for measurements. The temperature was kept constant at 30° C. with an electric stage warmer. A liquid filter of ferrous ammonium sulphate prevented heat from the lamp from reaching the object. The cells, thus, were disturbed as little as possible.

1 2

FIGURES 1 and 2. Camera lucida drawings of living spermatocytes of Chorthophaga. Side view of primary and secondary spermatocyte metaphases.

To measure the movement of chromosomes a metaphase in side view was selected, and as soon as the chromosomes began to separate, the distance between kinetochores was recorded at regular intervals with a camera lucida. In primary spermatocytes a bivalent with terminalized chiasmata near the spindle axis was chosen. In the secondary spermatocytes a chromosome in a median optical section of the spindle was selected. Though the spindle itself is hardly visible, it is clearly outlined by the chondriosomes (Figs. 1, 2). This makes it possible to measure the length and the equatorial diameter of the spindle during the entire anaphase. The various distances were then plotted against time, yielding a curve which describes the movement of the chromosomes and the changes in spindle length and diameter. All measurements were made with a 4 mm. Zeiss apochromat and 15 × ocular.

OBSERVATIONS

Anaphase movement in the first spermatocyte division

In Figure 3, three out of thirteen measured cells are presented.[2] The distance between separating kinetochores is plotted against time. The resulting curve con-

[2] The curves from different cells, even coming from different individuals, agree remarkably well, especially in the beginning of chromosome movement before spindle stretching sets in. The three cells shown indicate the degree of variation.

sists of an initial slow movement, then a straight portion of maximum velocity and a less regular part of gradually decreasing movement. Finally, before the cleavage furrow appears, the chromosomes move together again for a short distance, apparently due to the shrinkage of the spindle.

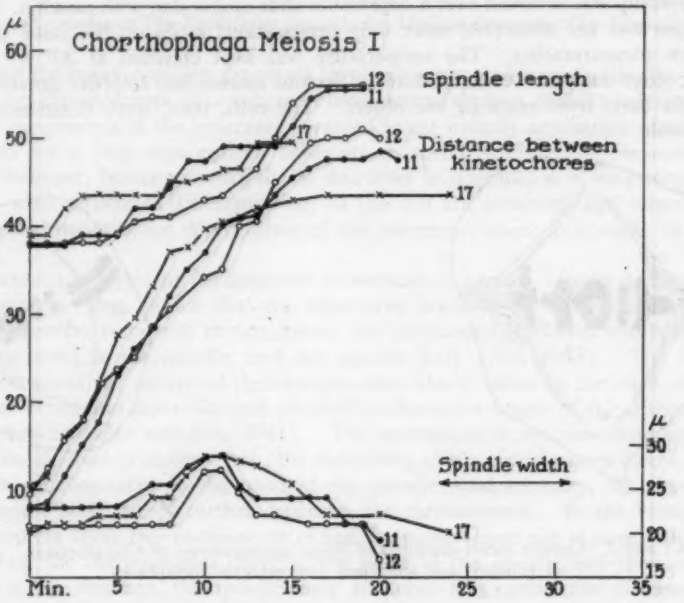


FIGURE 3. Chromosome movement and spindle behavior in the first meiotic division of *Chorthophaga viridifasciata*. See text.

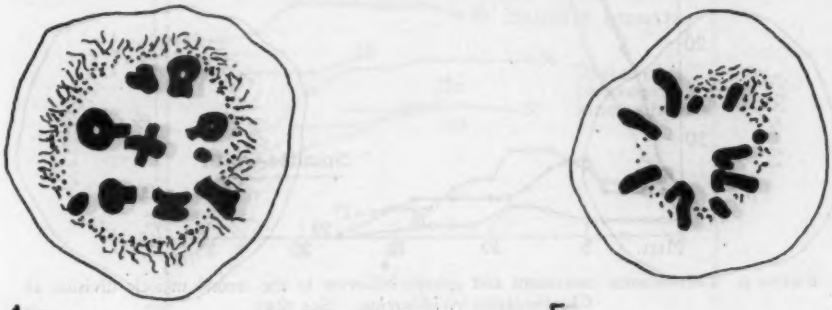
The spindle becomes visible in prometaphase through the alignment of the filamentous chondriosomes on its surface. In polar view their optical cross sections outline the spindle around its circumference (Fig. 4). In side view they appear lined up from the poles to the equator where they flow out into the equatorial plane (Fig. 1). Later, when the spindle elongates, the chondriosomes are stretched tightly on the spindle surface.

In Figure 3 the length of the spindle and its equatorial diameter are plotted against time during anaphase. We see how the spindle begins to elongate a few minutes after the onset of chromosome movement, contributing to their separation. There are thus two simultaneous processes involved in the later part of anaphase, namely, (1) the movement of the chromosomes to the poles due to the shortening of the chromosomal fibers, and (2) the elongation of the spindle. The diameter of the spindle increases in the later part of the chromosome curve, when the movement becomes irregular and slows down. Then it progressively decreases until the cleavage furrow cuts the spindle body in half. The spindle, therefore, increases

appreciably in volume during mid-anaphase. A comparison of the curves in Figure 3 shows that the spindle elongation varies more from cell to cell than the shortening of the chromosomal fibers. This indicates a greater sensitivity of that process to external conditions.

Anaphase movement in the second spermatocyte division

In the first division the chromosomes are distributed through the spindle body (Fig. 4). In the second division, however, they are oriented with their kinetochores at the periphery of a "hollow" spindle, the long arms pointing outwards (Fig. 5). Figure 6 gives the curves of three out of fifteen measured cells.[2] The



4 5

FIGURES 4 and 5. Camera lucida drawings of living spermatocytes of Chorthophaga. Polar view of primary and secondary metaphases.

movements of the chromosomes and the behavior of the spindle are much like those described for the first division. The chromosome curve is distinctly S-shaped. The spindle elongates a few minutes after the chromosomes have separated and increases in diameter in the later part of anaphase. Again there is thus a great increase in volume of the spindle. The rate of chromosome movement and spindle stretching is appreciably greater than in the first division.

In both divisions then we find the same type of anaphase movement. It begins with a shortening of the chromosomal fibers moving the chromosomes towards the poles. While this is continuing, the spindle begins to stretch, adding to the chromosome movement. Towards the end of anaphase the spindle begins to increase in width and then gradually shrinks until the cleavage furrow cuts it in half.

Experimental separation of the factors of anaphase movement

In the Hemiptera and Homoptera the action of chromosomal fibers and spindle elongation represent two distinct processes separated in time (Ris, 1943). In the grasshopper there is no such independence; the two processes are simultaneous and so neatly interwoven that a smooth movement of the chromosomes ensues. Is it possible to separate them experimentally? Methods have long been known which inhibit or destroy the spindle, such as ether, chloralhydrate, colchicine, etc. Is

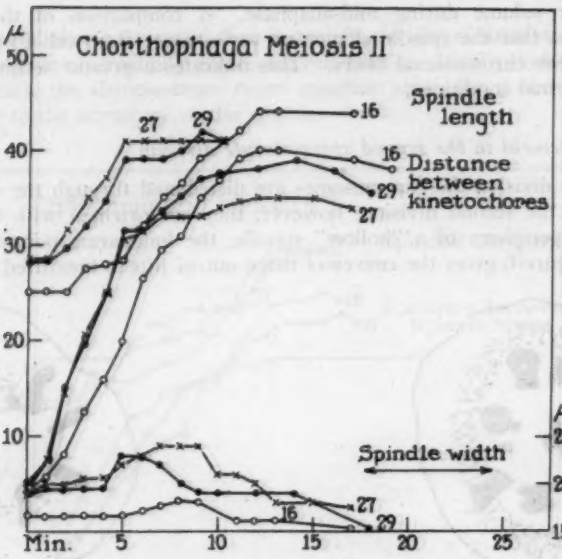


FIGURE 6. Chromosome movement and spindle behavior in the second meiotic division of *Chorthophaga viridifasciata*. See text.

there an agent which would inhibit one of the two processes without affecting the other? Colchicine, if added to the medium, either destroyed the spindle completely, or in lower concentrations had no effect on anaphase movement. Chloralhydrate, on the other hand, proved more useful. In concentrations higher than 0.1 per cent the spindle became shorter and narrower, and finally disappeared. The chromosomes were scattered irregularly through the center of the cell. The chondriosomes lost their regular orientation and began to penetrate between the chromosomes. At a concentration of 0.08 per cent cells were found in which the chromosomes moved to the poles, but where spindle elongation was inhibited. This seems to happen only within narrow limits of concentration of chloralhydrate inside the cell. If there is too much, the spindle will break down; if there is too little, it will elongate normally. This critical concentration is usually obtained only in a few cells of one cyst. The distance between chromosomes, and the length of the spindle, were then recorded during anaphase in primary spermatocytes exposed to chloralhydrate. In Figure 7 two such curves are shown (36 and 37). The spindle remained the same length all through anaphase in cell 37 and became only slightly longer in cell 36. The chromosomal fibers, on the other hand, must have remained active since the chromosomes had moved to the poles in a regular fashion. It can be shown that this action of the chromosomal fibers is normal. If we subtract the spindle elongation from the chromosome curve of an untreated primary spermatocyte, we obtain a curve which represents the movement of the chromosomes due to the chromosomal fibers alone. Two such curves are plotted in Figure 7 (13a and 17a). Since they agree well with the experimental curves (36 and 37), we must conclude that the

action of the chromosomal fibers was not affected by the chloralhydrate even though the spindle was prevented from elongating.

In the Hemiptera and Homoptera the two factors of anaphase movement, contraction of the chromosomal fibers and elongation of the spindle body, are separated in time. In the grasshopper they overlap in time, but their differing sensitivity to chloralhydrate has made it possible to separate them experimentally, inhibiting

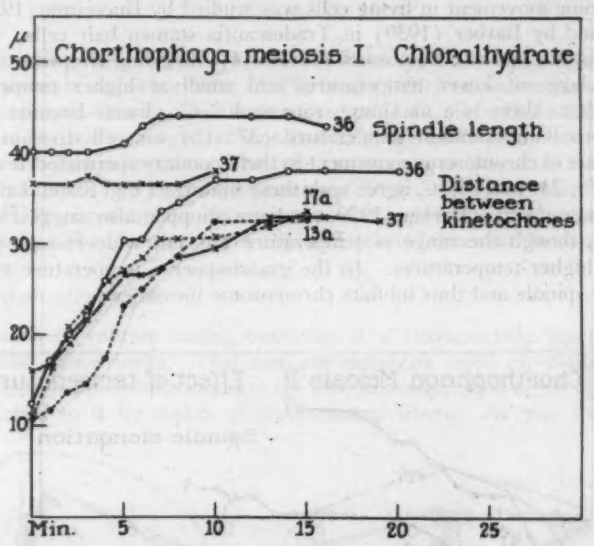


FIGURE 7. Chromosome movement and spindle elongation in Belar's solution with 0.08 per cent chloralhydrate (curves 36 and 37). Curves 13a and 17a represent the normal chromosome movement after the spindle elongation has been subtracted. The four curves are similar, showing that the movement to the poles in the absence of spindle stretching is normal.

spindle elongation without affecting the contraction of the chromosomal fibers. The difference between the grasshopper and the Hemiptera and Homoptera lies mainly in the relative timing of the component processes. In the grasshopper, spindle elongation sets in before the chromosomes have reached the poles. In the Hemiptera and Homoptera the spindle does not stretch until a few minutes after the poleward movement of the chromosomes has been completed.

Recently Callan (1941) described a case in which a separation of the components of anaphase movement occurs under natural conditions. In a trisomic grasshopper (Mecostethus) the unpaired extra chromosome sometimes moves into the equatorial plane during the first meiotic anaphase. In these cases the spindle does not elongate. The poleward movement of the chromosomes, however, does not seem to be disturbed. For reasons unknown, spindle elongation is inhibited under these conditions while the chromosomal fibers do not seem to be affected. Of course, we do not know here whether the rate of movement is normal as was shown in the chloralhydrate experiments.

The effect of temperature on the anaphase movement of chromosomes

The effect of temperature on mitosis has been repeatedly investigated, in most cases, however, on over-all processes such as the length of the mitotic phases, the rate of cleavage, etc. (see Belehradek, 1935). Only little can be concluded from such studies unless the processes are broken down into their components and the effect of temperature on these components analyzed. The effect of temperature on chromosome movement in living cells was studied by Bucciante (1927) in chick fibroblasts and by Barber (1939) in Tradescantia stamen hair cells. They found an increase in the rate of chromosome movement with rising temperature. This increase was large at lower temperatures and small at higher temperatures. In chick fibroblasts there is a maximum rate at 40° C. Fauré-Fremiet (1925) had earlier reported an optimum temperature (37° C.) for cell division in Ascaris. Measurements of chromosome movement in the secondary spermatocyte of the grasshopper at 17°, 23° and 30° C. agree with these findings (Fig. 8 and Table I). The data for Tradescantia (Barber, 1939) and grasshopper also suggest an optimum temperature, though the range of temperature was not wide enough to show the decrease at higher temperatures. In the grasshopper a temperature above 32° C. destroys the spindle and thus inhibits chromosome movement.

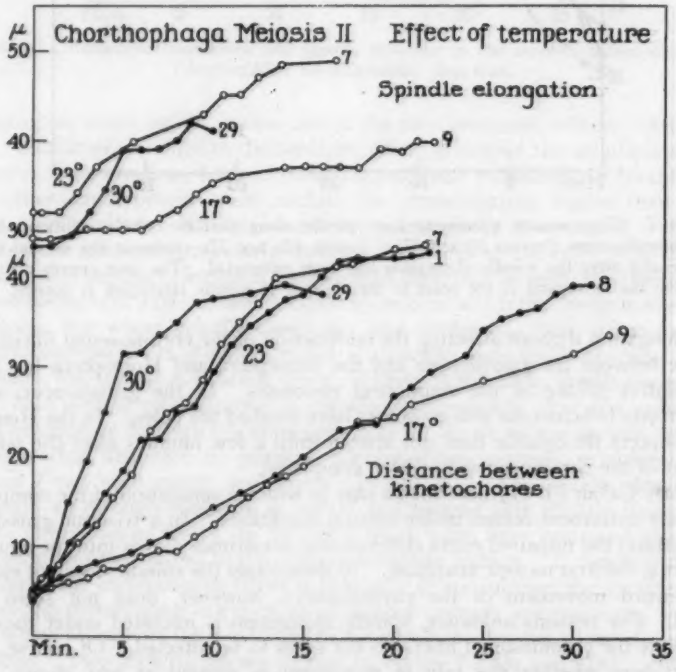


FIGURE 8. Chromosome movement and spindle elongation at 17°, 23°, and 30° C.

TABLE I

Effect of temperature on the rate of chromosome movement (chromosomal fibers only), and on the rate of spindle elongation. Micra/minute

Temperature	Maximum velocity chromosomes	Maximum rate spindle elongation
17° C.	0.4	1.4
23°	1.2	2.4
30°	2.5	3.6

Exposure to low temperature (1°–5° C.) destroys the mitotic spindle, as has been known since the experiments of O. Hertwig (1890) on sea urchins. If grasshopper spermatocytes at metaphase are exposed to 1° C., the spindle disappears, the chondriosomes become arranged at random, and the chromosomes are dispersed through the former spindle area. The cells can remain in this state for hours. If they are again exposed to a higher temperature (30° C.) the spindle forms anew, the chondriosomes are lined up on its surface and the chromosomes arranged in the metaphase plate. This process can be repeated several times on the same cells.

Abnormal spindle elongation

The increase in volume during anaphase is a characteristic property of the spindle in most animal cells. This swelling manifests itself especially in a pronounced elongation which contributes to the anaphase separation of the chromosomes that are attached to it by means of chromosomal fibers. As was shown above,

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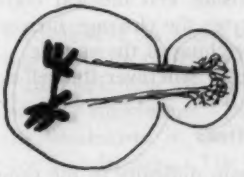

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FIGURES 9–12. Diagrams demonstrating the abnormal lateral stretching of the spindle in primary spermatocytes after X-ray-induced sticking of the chromosomes.

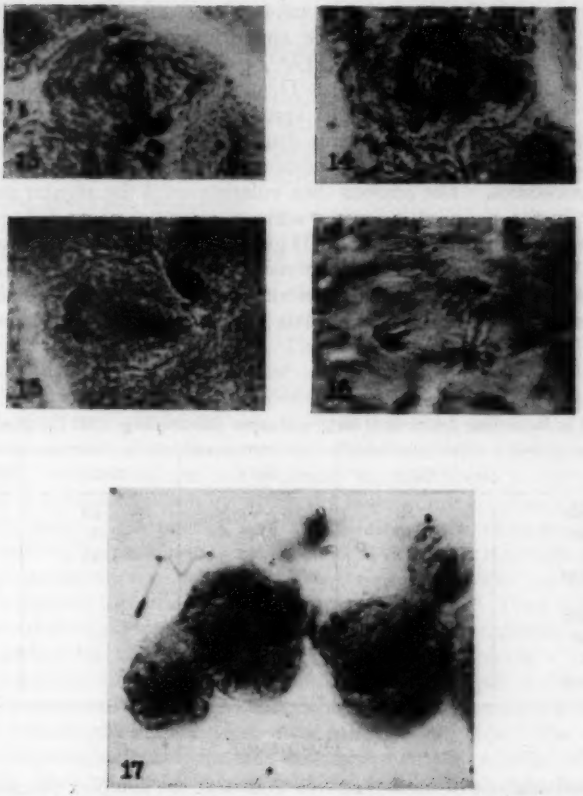
the spindle also increases in width in mid-anaphase and then gradually shrinks until it gets pinched through by the cleavage furrow. This stretching ability of the spindle is especially impressive under certain abnormal conditions. If the first meiotic division is observed in living cells after X-raying, in hypertonic medium or at temperatures around 32° C., one finds that chiasmata have a tendency to stick so that bivalents can not separate at anaphase. When the spindle begins to stretch, its normal elongation in the polar axis is inhibited by the combination of chromosomal fibers and sticking chromosomes. The spindle then begins to bulge in the equator, opposite the sticking chromosomes (Figs. 9, 14). If several bivalents fail to separate, the spindle may bend outward in several places (Figs. 10, 16). With the further elongation of the spindle, these lateral bulges become long and narrow projections which begin to push out the cell membrane. The poles of the spindle approach each other during this process, probably because of the action of the chromosomal fibers, which, instead of pulling the chromosomes to the poles, now draw the poles closer together (Figs. 11, 15). In fixed and stained preparations, the course of the continuous fibers shows clearly that the lateral projections are parts of the spindle bending outwards (Figs. 13–15). If chromosomes stick only on one side of the spindle, a very characteristic bent spindle results, looking like a spindle folded in the middle. Actually the origin is quite different, as described above. Sometimes the sticking chromosomes separate in mid-anaphase. The spindle then is able to assume its normal shape. It elongates in the polar axis and the lateral bulges disappear.

Spindle elongation and cleavage furrow

The relation of spindle elongation to cytoplasmic division, as demonstrated by these abnormal anaphases, is of special interest. When the spindle does not elongate normally, the cleavage furrow is always delayed or does not appear at all. More striking are the cells in which the spindle has been forced to elongate laterally in the equatorial plane. The lateral bulges of the spindle begin to push the cell out into long narrow processes (Figs. 11, 14). At the time when normally the cleavage furrow is formed, constrictions become visible around these cell projections. Often these constrictions develop into regular cleavage furrows and pinch off one or more small anuclear buds (Figs. 12, 17). In the cysts with secondary spermatocytes, one finds then cells with the diploid number of chromosomes which undergo the second division, and anuclear buds which do not divide any more. In grasshopper spermatocytes the cleavage furrow is therefore dependent on cell elongation caused by the stretching of the spindle. The location of the furrow is not predetermined, but can occur wherever the cell is pushed out.

Time relations

The main difficulty in the timing of the phases of mitosis, particularly in living cells, is the separation of the process into clearly delimited sections. The usual separation into prophase, metaphase, anaphase and telophase is not well suited for this purpose since the beginning or end of these phases is usually without sharp boundary. The duration of the following well-marked phases was measured in the spermatocytes of the grasshopper at 30° C.: First division: (1) Metakinesis, from



FIGURES 13–15. Anaphase in primary spermatocytes of Chorthophaga after irradiation with X-rays (100 r). Note the sticking of chromosomes and the lateral expansion of the spindle. Fixation: Sanfelice; stain: Iron-hematoxylin. 4 mm. Zeiss Apochromat, 15 × ocular. Compare with Figures 9–12.

the disappearance of the nuclear membrane to the formation of the metaphase plate. (2) Metaphase, from the establishment of the metaphase plate to the beginning of anaphase separation. (3) Ana-telophase, from the beginning of chromosome (kinetochore) movement to the appearance of the nuclear membrane. Interphase: from the formation of the nuclear membrane to its breakdown in the secondary spermatocyte. Second division: same phases as in the first division. The processes which mark these stages are clearly visible in the living cell. In the first division the asters are visible mainly due to the radial arrangement of the chondriosomes, but sometimes astral rays can be seen. The nuclear membrane, which was sharply outlined in prophase, becomes irregular and wrinkled, then disappears first near the asters. Wrinkled remnants can be seen for a few minutes before they vanish.

The metaphase spindle then slowly takes shape after the orientation of the asters. Its outline is marked by the chondriosomes. The spindle is at first rather narrow and short (cf. Belar, 1929, p 433). The chromosomes are thus crowded into the middle of the cell. Then, while the chromosomes become arranged into the metaphase plate, the spindle increases in width and in length. During metaphase the spindle remains constant in size and varies little from cell to cell. In telophase the nuclear membrane appears around a light area containing the chromosomes. The nucleus then enlarges until the regular interphase size is reached. In the second division the same processes are repeated, except that no asters can be seen in living cells. Table II gives the duration of these phases. Cells 1 and 2 were followed through the two divisions, cells 3 and 4 only through part of meiosis. Cells 3 and 4 are from a different individual than cells 1 and 2. At constant temperature the length of each phase varies only slightly from one individual to another.

TABLE II

Time relations in the meiotic divisions of the grasshopper Chorthophaga (30° C.) (hours and minutes)

	Cell 1	Cell 2	Cell 3	Cell 4
I. Metakinesis	50	—	43	—
I. Metaphase	2/30	2/45	2/40	—
I. Anaphase Telophase	1/25	1/40	1/25	—
Interphase	2/15	2/40	—	—
II. Metakinesis	25	—	—	25
II. Metaphase	1/25	1/25	—	1/35
II. Anaphase Telophase	1/45	1/47	—	1/45

DISCUSSION

The causal analysis of mitosis strives to dissect the complex process of cell division into its component factors and to elucidate their composition and their mode of action. Since Belar's classical study, the structures involved in the mitotic movements have again received deserved attention. In the grasshopper we can distinguish the following mitotic organelles: center, kinetochores, chromosomal fibers, spindle body.

The center

Like most animal cells the grasshopper spermatocytes contain a pair of centrioles which move to opposite sides of the nucleus in prometaphase and form the poles of the developing spindle. The asters are rather inconspicuous as in other cells with relatively little cytoplasm. In living cells they can be seen in prophase and occasionally in metaphase, especially in a hypertonic medium (cf. Belar, 1929). In secondary spermatocytes asters are even less distinct. Little is known about the function of the centers, except that they are probably involved in the organization of the spindle and in the cytoplasmic streaming which goes on during metaphase and anaphase.

Kinetochores

This specialized region of the chromosome is essential for the regular movements within the spindle. Fragments which are devoid of it lag behind and do not show any regular orientation, but may be moved passively by the stretching spindle or cytoplasmic currents on the surface of the spindle (White, 1935, 1937; Carlson, 1938). The main function of the kinetochores, perhaps in cooperation with the spindle or centriole, appears to be the formation of chromosomal fibers. Without kinetochores no chromosomal fibers can be formed.

Chromosomal fibers

Soon after the nuclear membrane has disappeared and the spindle begins to take shape, we can find in fixed and stained cells a distinct fibrous connection between the kinetochores and the spindle poles. These are the chromosomal fibers. They are usually not visible in living cells and some investigators therefore deny their existence.[3] Yet there is enough circumstantial evidence to show that they exist as differentiated structures within the spindle, and that they are the major factor in the anaphase movement of chromosomes (cf. Cornman, 1944; Schrader, 1944).

Belar (1929) emphasized the role of these "traction fibers." He assumes that they originate as a fluid secretion by which the kinetochore attaches itself to a fiber of the spindle body ("Leitfaser") and which allows the chromosome to glide along this "Leitfaser" in anaphase. Schrader (1944) accepts this view of Belar and bases on it his classification of spindles. Yet, the present writer could find no evidence for this indirect formation of the chromosomal fibers. They appear in prometaphase even before the spindle is fully formed as direct connections to the centers. They anchor the chromosomes to the poles of the spindle. So, when the spindle elongates, the chromosomes are carried along, the pull being transmitted through the chromosomal fibers to the kinetochores. If a chromosome sticks at anaphase, it will prevent the spindle from elongating on that side. The combination chromosome-chromosomal fibers is thus stronger than the spindle, while the cell membrane, for instance, yields to its pushing force. The chromosomal fibers, when they contract at anaphase, can even pull the spindle poles together and force the spindle out to one side of the cell (Figs. 9–12). The chromosomal fibers must thus be of greater consistency than the spindle body.

The spindle body

In grasshopper spermatocytes the spindle body develops from nuclear material between the two centers. The area around the chromosomes remains distinct even after the nuclear membrane has disappeared, and stays free of cytoplasmic inclusions like chondriosomes. In metaphase the spindle is a viscous body which can be moved about and dissected out by microneedles (Chambers, 1924). It appears homogenous in the living cell and fibrous after fixation. The spindle is essential for the orientation of chromosomes and for the action of chromosomal fibers since they are anchored at its poles. The spindle can be destroyed by a number of agents: colchi-

[3] Chromosomal fibers are sometimes visible in forms with diffuse kinetochore, if the chromosomes are viewed on end and the light therefore has to pass the entire length of the sheet-like chromosomal fibers (Hughes-Schrader and Ris, 1941; Ris, 1942).

cine, chloralhydrate, cold, heat, hypertonic medium, etc. At the same time the regular arrangement of the chromosomes disappears and all chromosome movements are stopped.

The most striking action of the spindle is the elongation during anaphase. Belar (1929) found that in hypertonic media this elongation appears to be greatly exaggerated, and this led him to a very detailed study of spindle stretching in hypertonic solutions. His conclusions are briefly: (1) The spindle has a tendency to stretch; this tendency is exaggerated in hypertonic media. (2) The spindle, by origin, is differentiated into two half spindles, the "Stemmkörper" (pushing body) developing at anaphase between the daughter plates. (3) At anaphase, it is the "Stemmkörper" in particular which elongates.

As was shown above, abnormal spindle stretching occurs not only in dehydrated cells, but always when daughter chromosomes are made to stick together. A hypertonic medium is just one way of causing chromosomes to stick at anaphase. This effect of hypertonic solutions on chromosomes was described by Konopacki (1911) in cleavage divisions of echinoderm eggs, by Kostanecki (1898) in Myzostoma, and by Moellendorff (1938) in tissue cultures. Similar accidents are found at high temperatures (over 30° C.) and after exposure to X-rays. It is therefore not the hypertonic medium which induces the abnormal spindle stretching, but the resistance to elongation in the main axis, brought about by the sticking of chromosomes. From Belar's figures, it is obvious that in primary spermatocytes all the abnormal spindles are correlated with sticking chromosomes. The bent spindles in secondary spermatocytes are of a different and less extreme kind (Fig. 45, Belar, 1929). Here it seems to be the cell membrane which offers resistance to the elongating spindle and causes it to bend. X-ray-induced bridges cause the same kind of abnormal spindles in secondary spermatocytes as Belar described in the first division. Lateral expansion of the spindle after X-ray-induced chromosome sticking was also figured by White (1937, Figs. 12, 13).

But Belar figures some cells which show exaggerated elongation of the spindle without sticking of chromosomes. These cells had been treated in anaphase. During anaphase the spindle increases not only in length, but also in volume. Belar believed that the volume remained constant, though he did not commit himself definitely. His beautiful drawings, however, indicate quite clearly the swelling of the spindle which measurements have now substantiated. In hypertonic solutions the cell shrinks greatly, and as Belar pointed out, the cytoplasm more so than the spindle. The swelling of the spindle, then, encounters resistance, and it is probably this factor which causes the spindle to be longer, but narrower than normally. Even so, these spindles are only found in free floating cells. In this writer's preparations where the cells remained in the follicles, they did not occur. Another case of spindle stretching without chromosome sticking is found if prometaphases are treated with hypertonic solutions (Belar, 1929, Fig. 55; Ris, 1942, in spermatocytes of the bearberry aphid). Here again the spindle increases in width during its formation. In dehydrated cells this lateral growth is interfered with, and the spindle becomes long and narrow. In this connection it is important to note that the volume of the abnormally stretched spindles appears to be not larger than in normal spindles. There is, therefore, only a distortion in shape, not an actual increase in the spindle material.

Two factors then cause abnormal spindles: interference with the increase in width in prometaphase and mid-anaphase, and interference with normal stretching during anaphase through the sticking of chromosomes.

From his studies of these abnormal spindles, Belar came to the conclusion that the part of the spindle between the separating chromosomes was mainly responsible for the stretching. He called it the "Stemmkörper" (pushing body) and distinguished it from the two half spindles between the chromosomes and poles. This subdivision of the spindle is, however, artificial and unjustified. Belar himself points out the uniformity in the aspect of the entire spindle. Fibers and clefts are continuous. The only difference in anaphase is the presence of chromosomal fibers in the cone-shaped region between the chromosomes and the poles. This is responsible for the darker appearance after staining. In Belar's Figure 41 the chromosomal fibers are especially clear. The "Stemmkörper" concept originated in the observation that the region between the daughter plates elongates more rapidly than the entire spindle. This appears so, not because this region is a special part of the spindle, but because the chromsomal fibers actually shorten during spindle elongation, pulling the chromosomes to the poles. In this way the impression of a special stem body between the daughter plates is produced.

Furthermore, Belar thought that the initial separation of the chromosomes through action of the traction fibers releases the tension in the spindle and originates the action of the "Stemmkörper." But actual timing has now shown that the chromosomes travel a good distance to the poles before the spindle elongates. Besides, spindle stretching can occur without any action of the chromosomal fibers as is shown in the first spermatocyte division of Tamalia. The chromosomal fibers act merely as passive anchors for the chromosomes (Ris, 1943). In the lepidopteran Orgyia the spindle elongates though the chromosomes have no chromosomal fibers at all (Cretschmar, 1928, Figs. 48–50). All the evidence then indicates that there is no differentiation into "half spindles" and "Stemmkörper." The only real differentiations are the chromosomal fibers and the spindle body. The chromosomal fibers pull the chromosomes to the poles. The stretching of the spindle has nothing to do with this phase. It can go on just as well without spindle elongation (chloralhydrate experiment). But spindle elongation has its important functions. It separates the daughter plates still further by pushing the poles apart and thus indirectly moves the chromosomes anchored to them.

The picture of anaphase movement in grasshopper spermatocytes presented here is essentially in agreement with Belar's view. There is a "pulling action" of chromosomal fibers and the stretching of the spindle. But there are some modifications. The subdivision into half spindles and "Stemmkörper" is found to be artificial. The spindle as a whole elongates, at the same time increasing in volume. Its action on the chromosomes is indirect, through the chromosomal fibers which connect them to the spindle poles.[4] The chromosomal fibers are thought to connect the kinetochores directly to the poles without the intervention of a "Leitfaser." They shorten during anaphase and are alone responsible for moving the chromosomes to the spindle poles.

[4] Just how the chromosomal fibers are attached to the spindle is a very puzzling problem and nothing definite can be said about it at present.

In addition to being a major factor in the movement of chromosomes, the spindle body also seems to play a role in the division of the cytoplasm. If the spindle does not elongate, as in the chloralhydrate experiments, no cleavage furrow is formed. When the spindle stretches laterally instead of in its long axis, a cleavage furrow does appear at a right angle to this elongation in a quite unorthodox position and produces an anuclear bud (Figs. 9–12). Bauer (1931) illustrates a similar situation in spermatocytes of Tipula with abnormal spindles. His Figure 23 h suggests that it originated in the same fashion. Many examples can be found in the literature which show how the failure of spindle stretching causes absence of the cleavage furrow (for instance Dobzhansky, 1934; Callan, 1941). In most plant cells there is little or no stretching of the spindle and the cytoplasm is divided by the formation of a cell plate. But in the pollen mother cells of some plants a cleavage furrow is formed, and it is then associated with elongation of the spindle (Guignard, 1897; Farr, 1918). It appears then that in dividing cells, elongation of the cell and cleavage furrow are associated with spindle elongation (in contradiction to the unwarrantable generalization of Buchsbaum and Williamson, 1943). Dan has recently (1943) assembled convincing evidence that spindle elongation is the active agent in cell elongation and the following formation of a cleavage furrow.[5] On the other hand, in certain abnormal cases the spindle elongates and yet no cleavage furrow appears. The formation of a cleavage furrow clearly depends on other factors in addition to spindle elongation.

In a recent paper, Hughes and Swann (1948) published chromosome separation and spindle elongation curves for chick embryo cells in tissue culture. Chick chromosomes possess a localized kinetochore and the achromatic apparatus is similar to that of the grasshopper spermatocytes. The anaphase movement as described by the curves of Hughes and Swann is essentially the same as we found in the grasshopper. Their curves show spindle elongation to start right from the beginning of anaphase, while in the grasshopper it does not begin until the chromosomes have moved a considerable distance. This is probably not a real difference but the result of the great difficulties involved in making measurements in early anaphase on the small chromosomes and spindles of the chick embryo cells.

We have set out to describe the movement of chromosomes during anaphase in terms of the mitotic organelles involved. The structures responsible for this chromosome movement were found to be the chromosomal fibers and the spindle body. The chromosomal fibers move the chromosomes to the spindle poles by decreasing in length. The spindle body swells and stretches and moves the daughter chromosomes further apart, since they are anchored to the spindle by the chromosomal fibers. If these are broad sheets attached to the entire length of the chromosome (diffuse kinetochore), the spindle does not elongate until the chromosomes have reached the spindle poles (hemipteran and homopteran insects). If the chromosomal fibers are narrow bundles attached to a very short region of the chromosome (localized kinetochore), the spindle begins to stretch shortly after the chromosomes have begun to move. The two processes then act simultaneously producing a smooth unbroken chromosome separation curve. Though we cannot see here directly how the two factors act on the chromosomes, we can separate them experi-

[5] I am indebted to Dr. D. Costello, University of North Carolina, for making this paper available to me.

mentally by inhibiting spindle elongation with chloralhydrate. It is then possible to study the action of the chromosomal fibers alone.

Very little is known about the nature and mode of action of these organelles, and this aspect will not be discussed here. Many more exact data on the structure, composition, and behavior of spindle and spindle components under various conditions are needed before a fruitful hypothesis on the physico-chemical level can be brought forward.

Summary

The movement of chromosomes and the changes in spindle size have been recorded in living spermatocytes of the grasshopper during the meiotic divisions. Anaphase movement consists of two separate processes which are related to the action of distinct cellular organelles: (1) The shortening of chromosomal fibers moves the chromosomes to the poles. (2) The elongation of the spindle further separates the daughter plates. The two processes act simultaneously in the grasshopper. With chloralhydrate, spindle elongation can be inhibited without affecting the action of the chromosomal fibers. This demonstrates the independence of these two factors.

The effect of temperature on chromosome movement is shown by measurements at 17°, 23° and 30° C. Between 17° and 23° there is a greater increase in velocity of chromosome movement than from 23°–30° C. Temperatures above 32° C. inhibit mitosis through the destruction of the spindle.

Abnormal spindle elongation is found whenever chromosomes stick at anaphase. The spindle, unable to elongate in its long axis, expands laterally into a disc-shaped body which later forms one or several finger-like processes, pushing out the cell membrane. These lateral elongations usually give rise to one or more cleavage furrows, pinching off one or, rarely, more anuclear buds. This demonstrates clearly the relationship between spindle elongation, cell elongation, and cleavage furrow.

The role of the mitotic organelles in the anaphase movement of chromosomes is discussed. Indispensable for a regular anaphase are the kinetochores on the chromosomes, the chromosomal fibers, and the spindle body. No evidence was found for a specialized region in the spindle acting as "Stemmkörper." The spindle is uniform in structure and elongates uniformly.

Distinct recognition of the structures involved in anaphase movement, and a quantitative description of their function, forms a basis for experimental analysis of their composition as well as their mode of action.

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